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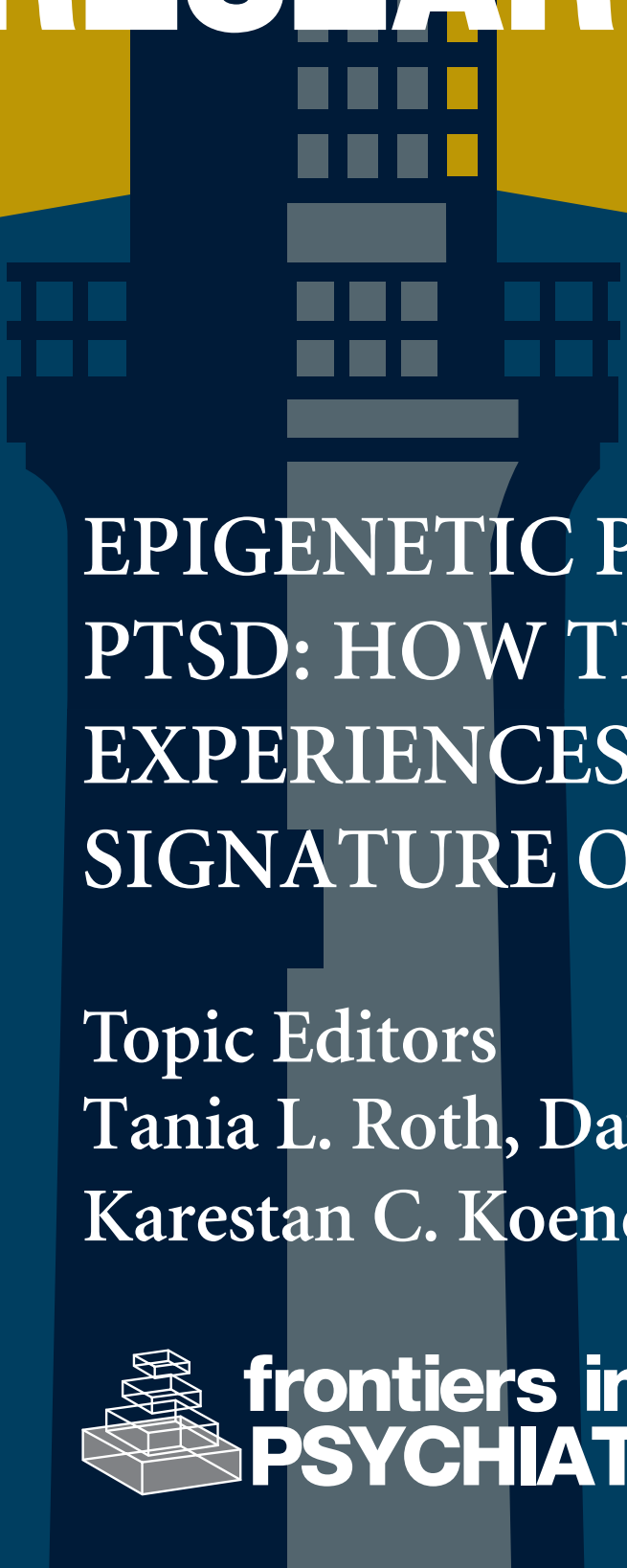

EPIGENETIC PATHWAYS IN PTSD: HOW TRAUMATIC EXPERIENCES LEAVE THEIR SIGNATURE ON THE GENOME

Topic Editors
Tania L. Roth, David M. Diamond and Karestan C. Koenen

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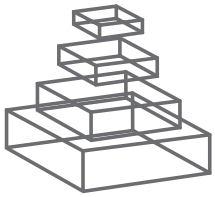

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EPIGENETIC PATHWAYS IN PTSD: HOW TRAUMATIC EXPERIENCES LEAVE THEIR SIGNATURE ON THE GENOME

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This research topic focuses on epigenetic components of PTSD. Epigenetic mechanisms are a class of molecular mechanisms by which environmental influences, including stress, can interact with the genome to have long-term consequences for brain plasticity and behavior. Articles herein include empirical reports and reviews that link stress and trauma with epigenetic alterations in humans and animal models of early- or later-life stress. Themes present throughout the collection include: DNA methylation is a useful biomarker of stress and treatment outcome in humans; epigenetic programming of stress-sensitive physiological systems early in development confers an enhanced risk on disease development upon re-exposure to trauma or stress; and, long-lived fear memories are associated with epigenetic alterations in fear memory and extinction brain circuitry.

Table of Contents

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EDITORIAL
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How traumatic experiences leave their signature on the genome: an overview of epigenetic pathways in PTSD

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Keywords: DNA methylation, histones, miRNA, stress, fear, PTSD

Epigenetic mechanisms are a class of molecular mechanisms by which environmental influences, including stress, can interact with the genome to have long-term consequences for brain plasticity and behavior. As PTSD, by definition, requires exposure to a traumatic event, and because genes are exquisitely sensitive to stress and trauma, epigenetic alterations have received attention as possible contributors to the development and persistence of PTSD symptoms. In this research topic, empirical support for the role of epigenetics in PTSD are presented and discussed. The articles assembled here cover a range of disciplines and experimental approaches in both animal models and humans that link stress and trauma with epigenetic alterations. Many articles also offer perspectives on epigenetics and sex differences, diagnosis and intervention, and future directions to bridge the gap between basic and clinical work.

Two original research articles provide empirical support for DNA methylation as a useful biomarker, not only in the detection and diagnosis of PTSD, but also as a biological measure for prediction of response to treatment, monitoring treatment efficacy, and prognosis of outcome. Rusiecki et al. (1) explore changes in DNA methylation of immune-related genes in US military service members with a PTSD diagnosis, showing differential patterns of methylation present pre- vs. post-deployment. Yehuda et al. (2) explore DNA methylation changes in association with changes in PTSD symptoms and other biological measures (including cortisol levels) in responders and non-responders to psychotherapy treatment.

Three review articles describe the compelling evidence for epigenetic alterations, particularly DNA methylation, as a consequence of exposure to stress encountered early in development. Raabe and Spengler (3) discuss studies showing early-life stress induced epigenetic alterations of stress genes as an important pathway in the dysregulation of stress systems in rodents and patients. Karsten and Baram (4) review the neuroanatomical and molecular pathways bridging sensory input with gene expression programing. They especially focus on how either nurturing or aversive early-life experiences can alter regulation of corticotropin-releasing hormone gene expression in hypothalamic neurons. McGowan (5) discusses studies of humans and animal model analogs that address molecular mechanisms underlying changes in stress-sensitive physiological systems in response to early-life trauma, paying particular attention to work on the glucocorticoid receptor. In an original research article, Kundakovic et al. (6) use a rodent model of early-life adversity (separation of infant mice from the mother) to explore the relationship between stress, genetic background, and sex in the determination of neurobehavioral and epigenetic outcomes. Together, data presented in these four articles are consistent with the notion that epigenetic programing early in life confers an enhanced risk on disease development upon re-exposure to trauma or stress. Throughout these articles, sex-specific differences at the epigenetic level are apparent too, suggesting that epigenetic activity plays an important role in sex-specificity and susceptibility to stress.

Continuing with a developmental theme, two review articles provide fascinating perspectives on the relationship between brain development and plasticity, gene × environment interactions, and the development of fear systems. Callaghan et al. (7) discuss developmental transitions in emotional learning and the role early-life stress has in both prematurely closing critical period plasticity and accelerating the development of fear learning systems. They also discuss the provocative idea of reopening critical periods of emotional learning to help treat many anxiety disorders. Nabel and Morishita (8) consider the potential contributions of "molecular brakes" identified in visual system development, the major model of critical period plasticity, to the development of fear system connections. They also discuss epigenetic regulators in the context of fear system development and their potential as new targets for therapeutic intervention.

One of the most common problems associated with PTSD is the persistence of memories of traumatic events. Since decades of research has shown that changes in gene expression occur when a memory is formed and stored, investigators have explored the relationship between DNA methylation and histone modifications and long-term trajectories in gene regulation associated with fear memories. These data are extensively described in two review articles. Maddox et al. (9) review the role of epigenetic mechanisms in animal models of fear learning and memory (Pavlovian fear conditioning paradigms that produce robust and long-lasting fear memories in rodents), highlighting epigenetic modulation of *FKBP5* in animal models of PTSD and clinical populations. Zovkic et al. (10) review literature supporting the involvement of epigenetics in PTSD, discussing data in the broader context of epigenetics in stress and fear learning. They also focus on evidence for epigenetic mechanisms as regulators of predisposition and resilience to PTSD, and provide a technical overview of approaches for measuring DNA methylation to encourage

future investigation of epigenetic mechanisms in animal models of PTSD.

Finally, though it is clear throughout this topic that DNA methylation has been the most extensively studied epigenetic alteration in outcomes associated with stress, evidence for histone modifications and microRNAs (miRNAs) as key epigenetic players are also emerging. Reul (11) discusses how psychologically stressful events evoke a long-term impact on behavior through changes in hippocampal function. Data are presented showing that this can occur through glutamatergic and glucocorticoid-driven changes in epigenetic regulation of gene transcription (via histone acetylation for example) within dentate gyrus neurons. In an original research article, Schmidt et al. (12) explore cortical miRNA expression profiles in a rodent model of PTSD. miRNAs are a more recent recognized class of epigenetic modulators of gene activity (or even a regulator of epigenetic processes), and are small non-coding RNAs that can regulate gene expression post-transcriptionally. Selective serotonin reuptake inhibitors (SSRIs) are the only FDA approved treatment for PTSD, with some evidence that one SSRI, fluoxetine, can ameliorate a subset of PTSD symptoms. These authors also examine fluoxetine effects on miRNA profiles, which may provide insight into the mechanisms underlying treatment effects of antidepressants in PTSD.

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ORIGINAL RESEARCH ARTICLE
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PTSD and DNA methylation in select immune function gene promoter regions: a repeated measures case-control study of U.S. military service members

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Background: The underlying molecular mechanisms of PTSD are largely unknown. Distinct expression signatures for PTSD have been found, in particular for immune activation transcripts. DNA methylation may be significant in the pathophysiology of PTSD, since the process is intrinsically linked to gene expression. We evaluated temporal changes in DNA methylation in select promoter regions of immune system-related genes in U.S. military service members with a PTSD diagnosis, pre- and post-diagnosis, and in controls.

Methods: Cases ($n = 75$) had a post-deployment diagnosis of PTSD in their medical record. Controls ($n = 75$) were randomly selected service members with no PTSD diagnosis. DNA was extracted from pre- and post-deployment sera. DNA methylation (%5-mC) was quantified at specific CpG sites in promoter regions of insulin-like growth factor 2 (IGF2), long non-coding RNA transcript H19, interleukin-8 (IL8), IL16, and IL18 via pyrosequencing. We used multivariate analysis of variance and generalized linear models to calculate adjusted means (adjusted for age, gender, and race) to make temporal comparisons of %5-mC for cases (pre- to post-deployment) versus controls (pre- to post-deployment).

Results: There were significant differences in the change of %5-mC pre- to post-deployment between cases and controls for H19 (cases: +0.57%, controls: −1.97%; $p = 0.04$) and IL18 (cases: +1.39%, controls: −3.83%; $p = 0.01$). For H19 the difference was driven by a significant reduction in %5-mC among controls; for IL18 the difference was driven by both a reduction in %5-mC among controls and an increase in %5-mC among cases. Stratified analyses revealed more pronounced differences in the adjusted means of pre-post H19 and IL18 methylation differences for cases versus controls among older service members, males, service members of white race, and those with shorter deployments (6–12 months).

Conclusion: In the study of deployed personnel, those who did not develop PTSD had reduced %5-mC levels of H19 and IL18 after deployment, while those who did develop PTSD had increased levels of IL18. Additionally, pre-deployment the people who later became cases had lower levels of IL18 %5-mC compared with controls. These findings are preliminary and should be investigated in larger studies.

Keywords: post-traumatic stress disorder, DNA methylation, cytokines, inflammation, promoter region, Operation Iraqi Freedom, Operation Enduring Freedom, epigenetics

INTRODUCTION

Estimates of post-deployment PTSD prevalence in U.S. military service members who served in Afghanistan and Iraq are 12 and 18%, respectively (Hoge et al., 2004); a recent study reported averages of 10–20% (Thomas et al., 2010). Despite these high rates among military populations, as well as high rates in civilian populations [i.e., U.S. prevalence rates of 7–8% (Kessler et al., 1994, 1997); lifetime prevalence of 9–14% (Kessler et al., 1995; Breslau, 2001)], the underlying molecular mechanisms of PTSD are largely unknown.

Given the sustained stress of the sympathetic nervous system and resulting hyper-arousal state in PTSD, it has been hypothesized that immune system functioning is affected. A series of recent studies has shown that PTSD is associated with alterations in immune function (Hoge et al., 2009) in particular increased levels of some canonical pro-inflammatory cytokines (Spivak et al.,

1997; Maes et al., 1999; Sutherland et al., 2003; von Kanel et al., 2007) and decreased levels of anti-inflammatory cytokines (Kawamura et al., 2001; von Kanel et al., 2007; Smith et al., 2011). Profiling using cDNA microarrays of peripheral blood during the triggering and development of PTSD in trauma survivors at the emergency room and 4 months later has found differential gene expression signatures in promoter regions of genes which distinguished PTSD patients (Segman et al., 2005; Zieker et al., 2007). Distinct expression signatures were found in particular for transcripts involved in immune activation (Segman et al., 2005). Up-regulated genes included insulin-like growth factor 2 (IGF2) and pro-inflammatory/neuroprotective chemokine, IL8 (CXCL8); down-regulated genes included the pro-inflammatory pleiotropic cytokines, interleukin 16 (IL16), and interleukin 18 (IL18) (Segman et al., 2005; Zieker et al., 2007). Similar findings of differential expression of genes involved in immune cell function were found among cases of PTSD from the World Trade Center attack versus controls (Sarapas et al., 2011) and in a study of Bosnian war refugees with PTSD versus controls (Nowotny et al., 2010).

An epigenetic mechanism, DNA methylation may play a significant role in the pathophysiology of PTSD, since the process is intrinsically linked to gene regulation. Recently, epigenetic mechanisms, including regulation of chromatin structure and DNA methylation, have been found to be regulators of gene transcription in the CNS (Zovkic and Sweatt, 2013). Methylation changes may completely silence a gene or may decrease or increase gene expression. It is hypothesized that epigenetic molecular mechanisms, especially DNA methylation/demethylation, may influence long-term behavioral change through active regulation of gene transcription in the CNS. A recent review proposed that epigenetic molecular mechanisms underlie the formation and stabilization of context- and cue-triggered fear conditioning based in the hippocampus and amygdala, a conclusion reached in a wide variety of studies using laboratory animals (Zovkic and Sweatt, 2013). In humans, there is a small, but growing body of literature that supports a potential link between DNA methylation of immune function genes and PTSD. A recent study applying methylation microarrays to assay CpG sites in peripheral blood from PTSD cases and controls found differential methylation in genes related to immune system functions (Uddin et al., 2010). In particular, this study found that IL8, a gene that regulates innate and adaptive immune system processes, was unmethylated among PTSD cases (Uddin et al., 2010). Another recent study in an African American population found that psychosocial stress may alter global and gene-specific DNA methylation patterns potentially associated with peripheral immune dysregulation (Smith et al., 2011).

In the present study, we focused on a set of growth factors/transcripts and cytokines/chemokines previously implicated in PTSD: paternally imprinted IGF2, maternally imprinted H19 (a long, non-coding RNA transcript), IL8, IL16, and IL18 (Segman et al., 2005; Zieker et al., 2007; Nowotny et al., 2010; Uddin et al., 2010; Sarapas et al., 2011; Smith et al., 2011). We evaluated promoter region methylation levels as percentage of 5-methyl cytosine (%5-mC) in DNA from serum of PTSD cases and controls, who were U.S. military soldiers who deployed to Afghanistan [Operation Enduring Freedom (OEF)] or Iraq [Operation Iraqi Freedom (OIF)] between 2004 and 2006. Our study had the unique ability to investigate temporal changes in methylation patterns after deployment [a proxy for exposure to a potentially traumatic event (PTE)]. The pre- and post-deployment samples collected in our study enable such evaluations of temporal changes in methylation patters. Our objectives were to investigate DNA methylation patterns associated with PTSD.

Since human studies of brain tissue are highly invasive, identifying a low-invasive biomarker of epigenetic patterns of PTSD would be of great clinical value. Most of the studies to date which have measured expression signatures or methylation patterns in PTSD or other psychiatric disorders have been carried out using peripheral blood (Segman et al., 2005; Zieker et al., 2007; Nowotny et al., 2010; Uddin et al., 2010; Sarapas et al., 2011; Smith et al., 2011). To our knowledge, serum has not yet been evaluated for gene-specific methylation patterns potentially associated with PTSD. Serum and cerebrospinal fluid (CSF) have been found to have good correlation with respect to cytokine gene expression, and in this exploratory study we evaluated DNA methylation from serum as a biomarker, without drawing links to other types of tissues or directly extrapolating its significance. Serum DNA methylation patterns may provide a surrogate indicator of differential response to stress and vulnerability to PTSD. Unlike previous studies, we had unique access to biologic samples prior to onset of disease and prior to deployment (a proxy for exposure to a PTE). Serum samples from military service members have been stored at the Department of Defense Serum Repository (DoDSR) since the early 1990's. We were able to access those stored sera and identified samples drawn prior to and post-deployment for all cases and controls.

MATERIALS AND METHODS

STUDY POPULATION

The study population has been described previously (Rusiecki et al., 2012). The target population was male and female U.S. Army and Marines service members serving their first OEF/OIF deployment between January 01, 2004 and December 31, 2006. Deployment length was between 6 and 18 months. Via query of medical records using the International Classification of Diseases, Ninth Revision (ICD-9) codes 290–320, we determined an absence of any mental health diagnoses dating back to at least 2 years prior to first OEF/OIF deployment for all cases and controls. In an effort to exclude possible confounding by other psychiatric illnesses, for which differential gene expression or methylation has been reported (Mill and Petronis, 2007; Shimabukuro et al., 2007; Tamura et al., 2007; Kuratomi et al., 2008; Feng and Fan, 2009; Iwamoto and Kato, 2009; Gavin and Sharma, 2010) post-deployment exclusion criteria for both cases and controls was ever having a health encounter for schizophrenia (ICD-9 code 295), bi-polar disorders (ICD-9 code 296), and manic phase bi-polar disorder (also ICD-9 code 296).

The PTSD cases ($n = 75$) had existing pre- and post-deployment serum samples housed at the DoDSR, met all the criteria above, and had at least two outpatient records with a primary diagnosis of chronic PTSD, based on ICD-9 Code 309.81 in the first diagnostic position. The first outpatient diagnosis was between 4 and 12 months after return from first deployment. The second outpatient diagnosis was any time after that, but within

2 years of return from first deployment. Additional criteria for inclusion as a case to this study was having one serum sample (the pre-deployment sample) drawn within 12 months prior to first OEF/OIF deployment and one sample (the post-deployment sample) drawn within 6 months after return from first OEF/OIF deployment. Cases were randomly selected within each gender, such that 25 females and 50 males were included. We identified an appropriate active duty service member control group ($n = 75$), who were frequency matched from a stratified, random sample based on age (20–26, 27–35), gender, and race, but for whom there was never a diagnosis of PTSD (ICD-9 Code = 309.81) or Traumatic Brain Injury (TBI) (ICD-9 Codes = 800.0–801.9, 803.0–804.9, or 850.0–854.1). Though cases in this study are presumed to have experienced a PTE by virtue of their PTSD diagnosis, the criteria of which requires exposure to a PTE, we have no data on formal assessment of trauma/PTE for either cases or controls. As such, this study uses deployment as a proxy for exposure to a PTE for both cases and controls. Because of Department of Defense policy regarding the DoDSR samples, we were unable to make contact with the study subjects for whom we had serum and medical encounter data.

SAMPLE PREPARATION AND LABORATORY METHODS

DNA extraction

For each PTSD case and control, The Armed Forces Health Surveillance Center identified a pre-deployment and a post-deployment serum sample and authorized release of up to 0.5 ml of serum per sample. These samples had been maintained at −30 °C continuously at the DoDSR. DNA was extracted using ChargeSwitch® gDNA 0.2–1 ml serum kit from (Invitrogen Carlsbad, CA, USA) and quantified via Qubit® dsDNA HS Assay Kit using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

Quantification of DNA methylation

DNA methylation was quantified via bisulfite treatment, PCR, and pyrosequencing. DNA was bisulfite treated using the Zymo DNA Methylation Kit (Zymo research, Orange, CA, USA). All assays used in this study were validated by PCR bias testing and sensitivity testing. This was done to assess the lowest input gDNA needed for bisulfite modification and the lowest input bisulfite converted DNA for PCR for each assay. For H19, IL8, IL16, and IL18, a minimum of 40 ng of DNA was used for bisulfite conversion. For IGF2, a minimum of 10 ng DNA was used for bisulfite conversion. The total DNA available for each sample was used as input gDNA for bisulfite conversion. Carrier RNA from Qiagen was used during the bisulfite modification and purification to minimize the loss of DNA during the procedure. Bisulfite treated DNA was eluted in 20 μl volume and 1 μl of it was used for each PCR. The PCR (45 cycles) was performed with one of the PCR primers biotinylated to convert the PCR product to single-stranded DNA templates. The PCR products (each 10 μl) were sequenced by Pyrosequencing PSQ96 HS System (Qiagen Pyrosequencing) following the manufacturer's instructions (Qiagen Pyrosequencing). Pyrosequencing is a real-time sequencing-based on mutation analysis or methylation analysis technology. The methylation status of each CpG locus was analyzed individually as a T/C SNP using QCpG software (Qiagen Pyrosequencing).

The loci of specific CpGs measured in each promoter region are shown in **Figure 1** and the rationale for selecting these specific loci was that they are generally located in the shore/vicinity of transcriptional start site. Loci measured were in the promoter regions of H19 [-1964, -1946, -1927, -1919 from the transcriptional H19 start site (TSS)], IGF2 [TSS-479, -476, -460, -361, -341, -322; note, the investigated regions for IGF2 covered the promoter of IGF2, p2, and the binding region for enhancer-blocking element CCCTC-binding factor (CTCF) upstream of the H19 start site], IL8 (TSS-116, -106, -31), IL16 (TSS-159, -139, -93, -79), and IL18 (TSS-158, -108, -86, -49, -33). The percentage of methylation (%5-mC) was expressed as 5-mC divided by the sum of methylated and unmethylated cytosine. This is interpreted as the percentage of cytosines at a given CpG site (or position) which is methylated. Percent 5-mC was measured at each CpG site, hereafter referred to as position, and a mean %5-mC was calculated across all positions measured in a promoter region. Starting from bisulfite modification, four controls [low, medium, high methylated DNA (EpigenDx, Inc.), and a no DNA template] were included in every pyrosequencing run to ensure specificity of PCR amplification, and success of pyrosequencing reactions.

DNA from cases and controls were randomly arranged on the plates for methylation quantification analysis. For 10% of the samples we included duplicates to which laboratory personnel were blinded, for the purpose of quality control (QC). Coefficients of variation as well as intraclass correlation coefficients (ICCs) were calculated for the means of each position in H19 (CV = 0.05, ICC = 0.75; $p < 0.01$), IL8 (CV = 0.01, ICC = 0.72; $p < 0.01$), IL16 (CV = 0.05, ICC = 0.68; $p < 0.01$), and IL18 (CV = 0.02, ICC = 0.89; $p < 0.01$). The duplicate samples were run on a separate plate from their counterparts, and some of the variation could be due to a plate effect.

Approximately 30% of the samples ($N = 86$) had DNA yields (e.g., <40 ng) insufficient for bisulfite conversion of H19, IL8, IL16, and IL18. Approximately 5% of the samples ($N = 15$) had DNA yields (e.g., <10 ng) insufficient for bisulfite conversion of IGF2. These samples were excluded from our study. For a small percentage of the samples for which bisulfite conversion was complete, pyrosequencing signals were too low to be reliable (0% for H19, 9% for IGF2, 0.05% for IL8, 8% for IL16, and 2% for IL18), thus we did not include them in our final analyses. It is unclear why signals were low for those particular samples, however, low signals were not associated with DNA yield. Distribution of samples either not measured or measured but with low signals did not differ by pre- and post-deployment or case/control status, with the exception of IGF2 pre-deployment case/control status. However, this is likely because there were only 16 with low signals for that assay, thus lending to the instability of those proportions.

Statistical methods

Our statistical analyses considered %5-mC in the promoter and/or known methylation region of each gene at each position (CpG site) measured as well as the mean of all the positions measured. We compared the change in cases' %5-mC from pre- to post-deployment, to the change in controls' %5-mC from pre- to post-deployment using multivariate ANOVA. We present the adjusted mean of pre-post methylation difference for cases versus

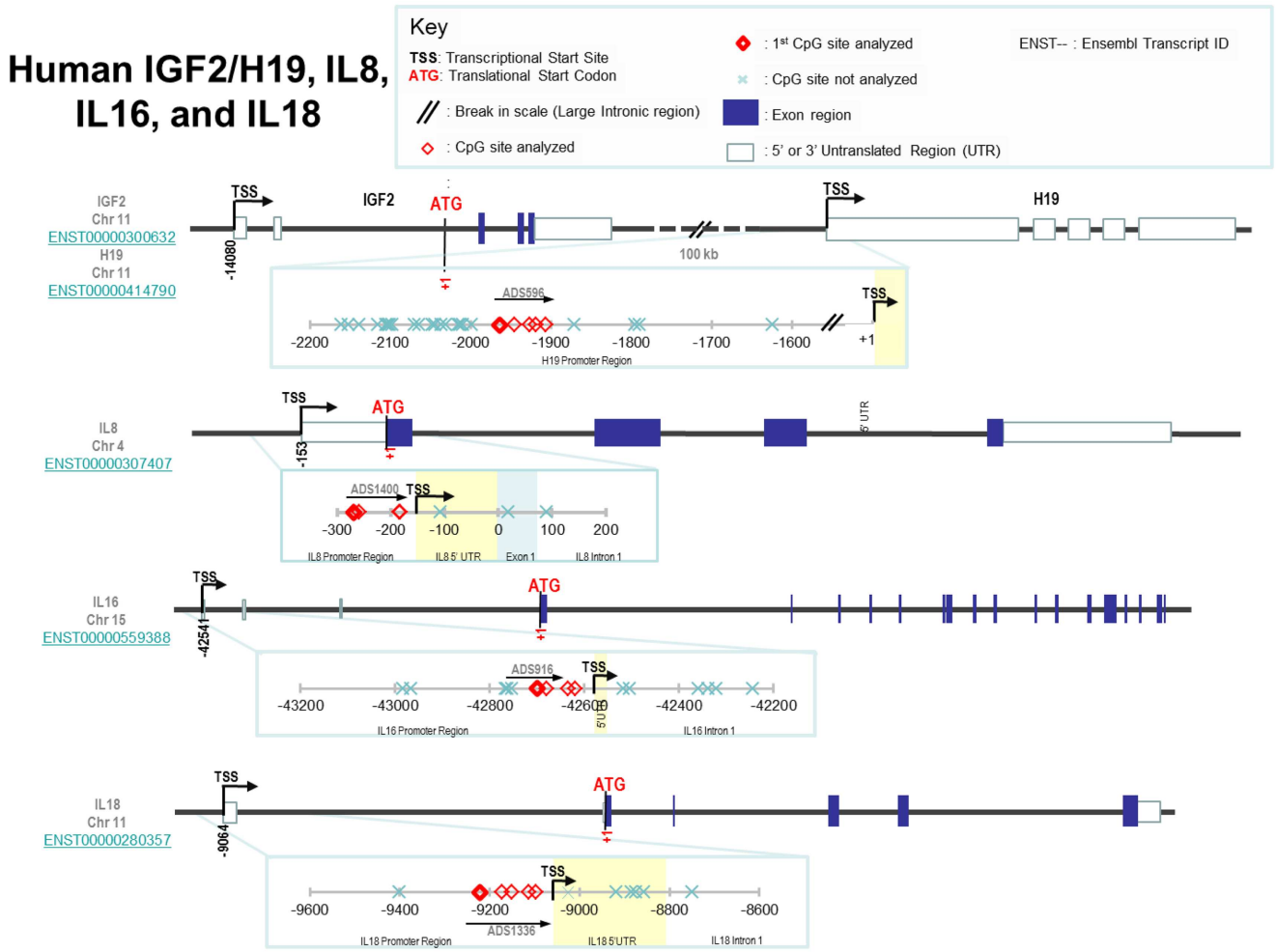

FIGURE 1 | Loci of specific CpGs measured in IGF2, H19, IL8, IL16, and IL18.

controls, adjusted for age, gender, and race. To further investigate the adjusted means and differences between cases and controls, pre-deployment and post-deployment, we ran generalized linear models (GLMs) Using GLMs, we carried out case-case and control-control (i.e., pre- to post-deployment) comparisons, as well as case-control comparisons, pre-deployment, and post-deployment. We carried out the same analyses, stratifying by age, gender, race, and length of deployment, separately, to investigate any potential patterns within specific groups. SAS Version 9.2 was used to carry out all statistical analyses. The SAS procedures GLM and ANOVA were used to run our final models (SAS Institute Inc., 2008).

This study was approved by the Institutional Review Board at the Uniformed Services University of the Health Sciences.

RESULTS

Baseline characteristics of the study population are in **Table 1**. Although the study was designed with 75 cases and 75 controls, we excluded 2 study subjects (1 case/1 control) because their DNA yield was insufficient for DNA methylation quantification for any of the loci measured in both pre- and post-deployment time frames. The remaining 148 study subjects did not necessarily have complete data for all loci measured in both pre- and post-deployment time frames, but they did have DNA methylation quantified for at least one locus in at least one time frame. The study population did not differ by case-control status for age, gender, and race because of the selection and frequency matching criteria. Approximately 73% of cases and 76% of controls had deployments of 6 months to less than 12 months, while 27% of cases and 24% of controls had deployments of 12–18 months. These small differences were not statistically significant. The number of days between end of deployment and post-deployment serum draw ranged from 1 to 170 (mean = 22.45; SD = 38.84; median = 7), and the number of days between pre-deployment serum draw and start of deployment ranged from 58 to 358 (mean = 87.46; SD = 84.49; median = 58.5). There were no differences between cases and controls for these time intervals, nor were there differences by occupational code or rank/rate. There were four cases who also had a post-deployment diagnosis of TBI (defined as an ICD-9 code of 800.0–801.9, 803.0–804.9, or

Table 1 | Baseline characteristics of population.

Characteristic	Cases ($N = 74$)		Controls ($N = 74$)		p-Value
	N	%	*N*	%	
AGE					
Younger (20–23 years)	40	54.1	38	51.4	0.74
Older (24–35 years)	34	46.0	36	48.7	
GENDER					
Male	49	66.2	49	66.2	1.00
Female	25	33.8	25	33.8	
RACE					
White	59	79.7	60	81.1	0.84
Black	15	20.3	14	18.9	
DEPLOY LENGTH					
Short (6 to <12 months)	54	73.0	56	75.7	0.71
Long (12–18 months)	20	27.0	18	24.3	
TIME BETWEEN DEPLOYMENT END AND POST-DEPLOYMENT SERUM SAMPLE DRAW					
≤7 days	41	55.4	38	51.4	0.62
>7 days	33	44.6	48	48.6	
TIME BETWEEN PRE-DEPLOYMENT SERUM DRAW AND DEPLOYMENT START					
≤90 days	52	70.3	45	60.8	0.23
>90 days	22	29.7	29	39.2	
OCCUPATIONAL CODING*					
Less likely involved in combat	19	38.8	20	38.5	0.97
More likely involved in combat	30	61.2	32	61.5	
Missing	25		22		
RANK/RATE#					
Junior enlisted	57	77.0	52	70.3	0.35
Middle-grade/senior enlisted and officer	17	23.0	22	29.7	

*Based on military occupational specialty POC coding as either less likely to be involved in combat or more likely to be involved in combat.

#Junior enlisted included E-2 through E-4; middle/senior enlisted and officer included E-5 through E-8, O-1 through O-3, and W-2.

850.0–854.1) (data not shown). We carried out all analyses including and excluding those four cases to evaluate potential confounding by TBI and found that results were very similar. We, therefore, present all results for analyses including those four PTSD cases with concurrent TBI.

Table 2 presents the results of a multivariate ANOVA comparing adjusted means of pre-post methylation differences for cases versus controls. These results show that, accounting for the exposure of deployment (i.e., proxy for PTE), there were significant reductions in %5-mC for controls, compared with cases at H19 (mean of positions 1–4: cases: +0.57%, controls: −1.97%; $p = 0.04$) and IL18 (mean of positions 1–5: cases: +1.39%, controls −3.83%; $p = 0.01$). Position 3 in H19 and positions 4 and 5 in IL18 had statistically significant differences between cases and controls, and though differences in the change of methylation were not significant at other positions for H19 and IL18, they all showed similar patterns.

Table S1 in Supplementary Material provides additional detail on the change in methylation across deployment for cases and controls; paired differences in adjusted mean levels for cases pre- and post-deployment and controls pre- and post-deployment, determined via GLM, are presented. The differences between cases and controls across deployment for H19 were driven primarily by the significant reduction in %5-mC among controls from pre- to post-deployment (the levels for cases were unchanged across deployment for H19). The pattern of decreased H19 methylation among controls from pre- to post-deployment is evident; statistically significant decreases were found at positions 1, 3, and 4, and for the mean of positions 1–4. The differences for IL18 were driven by both the decrease in methylation across deployment for controls as well as the increase in methylation across deployment for cases. There was a pattern of decreased methylation among controls from pre- to post-deployment, though a statistically significant decrease was found for position 5 only. There was also a pattern of increased IL18 methylation for cases pre- to post-deployment, though none of the comparisons was statistically significant.

Table S2 in Supplementary Material, which presents case-control comparisons for both pre- and post-deployment, further illustrates that for IL18, %5-mC was lower in cases compared with controls, pre-deployment ($p \leq 0.05$ for positions 2, 4, and 5, and the mean of the five positions), while the pattern reversed post-deployment, such that levels were non-statistically significantly higher in cases compared with controls. Note that the term "case"

Table 2 | Multivariate ANOVA comparing adjusted* mean of pre-post methylation difference between cases and controls.

Gene promoter		N_{pairs}[1]	Mean[2]	SE	*p*-Value
H19 (mean positions 1–4)	Case	37	0.57	1.10	0.04
	Control	45	−1.97	0.99	
Position 1; TSS-1964	Case	37	0.58	1.30	0.09
	Control	45	−1.92	1.16	
Position 2; TSS-1946	Case	37	1.38	1.41	0.14
	Control	45	−1.01	1.26	
Position 3; TSS-1926	Case	37	0.65	1.15	0.01
	Control	45	−2.93	1.03	
Position 4; TSS-1919	Case	37	−0.33	1.44	0.31
	Control	45	−2.01	1.29	
IGF2 (mean positions 1–6)	Case	52	−2.61	3.25	0.81
	Control	62	−3.49	3.01	
Position 1; TSS-479	Case	52	−0.22	4.77	0.76
	Control	62	−1.83	4.42	
Position 2; TSS-476	Case	52	−2.97	4.94	0.59
	Control	62	0.00	4.58	
Position 3; TSS-460	Case	52	4.16	4.93	0.40
	Control	62	−0.52	4.57	
Position 4; TSS-361	Case	52	−12.14	4.59	0.67
	Control	62	−9.98	4.32	
Position 5; TSS-341	Case	52	−2.79	3.97	0.84
	Control	62	−3.71	3.74	
Position 6; TSS-322	Case	52	−2.90	3.37	0.95
	Control	62	−3.13	3.17	
IL8 (mean of positions 1–3)	Case	36	−0.17	0.32	0.25
	Control	44	−0.58	0.28	
Position 1; TSS-116	Case	36	−0.54	0.54	0.38
	Control	44	−1.06	0.47	
Position 2; TSS-106	Case	36	−0.12	0.46	0.36
	Control	44	−0.58	0.40	
Position 3; TSS-31	Case	36	0.15	0.17	0.20
	Control	44	−0.10	0.15	
IL16 (mean of positions 1–4)	Case	31	−4.23	3.66	0.88
	Control	38	−3.61	3.66	
Position 1; TSS-159	Case	31	−5.61	4.80	0.97
	Control	38	−5.44	4.78	
Position 2; TSS-139	Case	31	−2.94	3.34	0.89
	Control	38	−3.44	3.33	
Position 3; TSS-93	Case	31	−4.29	6.58	0.95
	Control	38	−4.76	6.64	
Position 4; TSS-79	Case	31	−5.00	6.22	0.85
	Control	38	−3.78	6.27	
IL18 (mean of positions 1–5)	Case	37	1.39	1.85	0.01
	Control	45	−3.83	1.66	
Position 1; TSS-158	Case	37	−2.60	2.66	0.50
	Control	45	−4.63	2.40	
Position 2; TSS-108	Case	37	2.31	2.31	0.03
	Control	45	−3.50	2.08	
Position 3; TSS-86	Case	37	1.88	2.47	0.21
	Control	45	−1.66	2.22	
Position 4; TSS-49	Case	37	2.35	1.93	0.01
	Control	45	−3.43	1.75	
Position 5; TSS-33	Case	37	2.13	2.23	0.02
	Control	45	−4.03	2.02	

Adjusted for age, gender, race.

[1] Pairs of samples, i.e., pre and post paired samples among the cases and among the controls – number of pairs differ for the different promoter regions measured because of sensitivity of each assay – i.e., some samples were dropped because of low pyrosequencing signals for a specific assay.

[2] Adjusted mean of the difference from pre- to post-deployment for cases and for controls.

in the pre-deployment context refers to people who later became cases, post-deployment; during the pre-deployment time period, they were not yet cases.

Also shown in Table S2 in Supplementary Material are a few other loci where significant differences in %5-mC were found between cases and controls pre-deployment, i.e., IGF2 position 2 ($\%5\text{-mC}_{cases} > \%5\text{-mC}_{controls}$, $p = 0.01$) and IL8 position 2 ($\%5\text{-mC}_{cases} < \%5\text{-mC}_{controls}$, $p = 0.01$). There were no significant differences in adjusted mean levels for the post-deployment case-control comparisons.

Stratified analyses (**Table 3**) revealed more pronounced differences in the adjusted means of pre-post H19 and IL18 methylation differences for cases versus controls among older (24–35 years) service members (H19 position 3 case/control: +0.13/−3.66; $p = 0.05$), males (H19 position 3 case/control: +0.80/−2.99; $p = 0.01$; IL18 mean case/control: +0.47/−5.38), service members of white race (H19 mean case/control: +0.72/−2.70; $p = 0.01$; IL18 mean case/control: +2.82/−2.16; $p = 0.04$), and those with deployments between 6 and 12 months (H19 position 3 case/control: +0.16/−3.20; $p = 0.02$; IL18 mean case/control: +2.52/−4.65; $p = 0.01$).

DISCUSSION

Our study found that there were significant differences in the change of methylation (%5-mC) across deployment between cases and controls for H19 and IL18. While there was no change in %5-mC in H19 for cases from pre- to post-deployment, there was a significant decrease among controls driving the difference in %5-mC change between the cases and controls. For IL18 there was a significant difference between the %5-mC decrease in controls and the %5-mC increase in cases, from pre- to post-deployment. These findings were more pronounced in both H19 and IL18 in younger service members, males, service members of white race, and those with shorter deployment length. There were a few additional statistically significant findings at specific CpG sites, such as pre-deployment case-control differences in IGF2 position 2 and in IL8 position 2. Given the low signals from the IGF2 assay

Table 3 | Multivariate ANOVA comparing adjusted* mean of pre-post methylation difference for cases versus controls, stratified by age, gender, race, and deployment length.

Gene promoter/CpG site	Subject	Age: younger (20–23) and older (24–35)					
		Younger			Older		
		N	μ	*p*-Value	*N*	μ	*p*-Value
H19 position 3	Case	20	0.71	0.11	17	0.13	0.05
	Cont	25	−2.47		20	−3.66	
H19 mean of positions	Case	20	0.34	0.36	17	0.58	0.07
	Cont	25	−1.34		20	−3.00	
IL18 mean of positions	Case	20	0.60	0.22	17	1.38	0.05
	Cont	25	−3.94		20	−3.07	
		Gender: female and male					
		Female			Male		
		N	μ	*p*-Value	*N*	μ	*p*-Value
H19 position 3	Case	9	0.59	0.24	28	0.80	0.01
	Cont	16	−2.74		29	−2.99	
H19 mean of positions	Case	9	0.98	0.12	28	0.91	0.14
	Cont	16	−2.84		29	−1.27	
IL18 mean of positions	Case	9	1.12	0.35	28	0.47	0.03
	Cont	16	−1.80		29	−5.38	
		Race: black and white					
		Black			White		
		N	μ	*p*-Value	*N*	μ	*p*-Value
H19 position 3	Case	6	−0.65	0.43	31	1.64	0.01
	Cont	8	−3.07		37	−2.30	
H19 mean of positions	Case	6	−0.95	0.73	31	0.72	0.01
	Cont	8	0.14		37	−2.70	
IL18 mean of positions	Case	6	−0.31	0.21	31	2.82	0.04
	Cont	8	−5.53		37	−2.16	
		Deployment length: long (6 to <12) and short (12–18)					
		Long Depl.			Short Depl.		
		N	μ	*p*-Value	*N*	μ	*p*-Value
H19 position 3	Case	8	4.20	0.42	29	0.16	0.02
	Cont	11	0.75		34	−3.20	
H19 mean of positions	Case	8	3.56	0.38	29	0.12	0.12
	Cont	11	0.68		34	−2.14	
IL18 mean of positions	Case	8	−2.08	0.93	29	2.52	0.01
	Cont	11	−2.46		34	−4.65	

**For analyses stratified on age, adjusted for gender and race; for analyses stratified on gender, adjusted for age and race; for analyses stratified on race, adjusted for age and gender; for analyses stratified on deployment length, adjusted for age, gender, and race.*

being differentially distributed between pre-deployment cases and controls, we are cautious to interpret the results for IGF2. Whether these patterns represent markers of vulnerability or resiliency is speculative. Psychological stress incurred during deployment may be associated with a methylation response in these genes, but given the lack of PTE data for controls, this study is not able to address whether the response is protective of PTSD or puts one at risk for PTSD.

The most consistent result from our study, which is detailed in Table S2 in Supplementary Material, is that cases pre-deployment had significantly lower %5-mC in the IL18 promoter region than controls pre-deployment. This difference was found at positions 2, 4, and 5, and the mean of all five positions; *p*-values approached significance for positions 1 (0.08) and 3 (0.06). There was a reversal of this pattern post-deployment, in that cases had higher levels at all positions, compared to controls, though differences were not statistically significant. Given that DNA methylation is often inversely correlated with gene expression, in particular for immune system-related genes (Oliveira et al., 2009) these results for IL18 are generally consistent with findings from a previous study based on cDNA microarray investigation, which reported down-regulation of IL18 in PTSD cases (Segman et al., 2005). Interestingly, pro-inflammatory cytokine IL18, a key mediator of inflammation and immune response inducing interferon-γ (IFN-γ), is expressed in the brain and plays a significant role in a number of neuropathological disorders (Bossu et al., 2010; Anderson and Rodriguez, 2011). IL18 has been recently shown to attenuate breaks in the blood-brain barrier via IFN-γ independent pathway, suggesting its potential neuroprotective role (Jung et al., 2012). Our results of increased IL18 methylation in cases would imply down-regulation of the gene, which is consistent with previous findings (Zieker et al., 2007).

Our findings also suggest a potential role of H19 in stress response (23). The imprinted IGF2-H19 locus (CTCF binding cite) encodes the growth promoting hormone IGF2 and a long non-coding RNA H19. The maternally inherited locus transcribes untranslated H19 RNA, which plays the role of a trans-regulator of the imprinted gene network controlling embryonic growth and was recently shown to be processed into micro-RNA (miRNA)-675 (Cunningham et al., 2010; Keniry et al., 2012). Expression of both genes is partially regulated by a 3′ distal enhancer, and methylation of the CTCF binding site on the paternal chromosome prevents binding of the CTCF insulator allowing for activation of the IGF2 promoter. Lack of methylation of the CTCF binding site on the maternal chromosome, however, prevents IGF2 promoter activation (Fu et al., 2004; Cunningham et al., 2010; Keniry et al., 2012). Although a previous study reported that IGF2 was up-regulated in the whole blood (Zieker et al., 2007) IGF2-mediated signaling has been implicated in fear extinction and was proposed as a therapeutic venue to attenuate excessive fear memory potentially via promotion of neuronal survival and/or self-renewal of neural stem cells through its interaction with the insulin receptor (Agis-Balboa et al., 2011; Ziegler et al., 2012). This could suggest that it is not only IGF2 that may play a direct role in PTSD fear extinction, but H19 may be involved in the regulation of IGF2. Also, H19 or its miRNA products and inhibition of their putative targets may contribute to the stress response (23).

Previously, we investigated DNA methylation in repetitive elements, LINE-1 and Alu, in this population and found differential methylation between pre- and post-deployment controls and in cases versus controls, post-deployment. In the current study, we selected specific cytokine promoter regions of interest based on previous findings in the literature of differential gene expression or methylation of those cytokines between PTSD cases and controls (Segman et al., 2005; Zieker et al., 2007; Nowotny et al., 2010; Uddin et al., 2010; Sarapas et al., 2011; Smith et al., 2011). Increasing evidence for contribution by the chemokine network (Semple et al., 2010) and the known role of IGF2 (Torres-Aleman, 2010) in brain and vasculature development indicates it as an important target due to possible pre-morbid conditions. The neuro-chemokine IL8 has been implicated in brain development, neuroinflammation, and synaptic transmission (Rostene et al., 2011). Cytokine IL16, a chemo-attractant for immune cells expressing surface CD4 molecules (e.g., T-cells) (Cruikshank and Little, 2008) targets a number of neurophysiological membrane proteins, such as immediate-early gene c-Fos, potassium and calcium channels, *N*-Methyl-D-aspartate (NMDA)-receptor subunits, and neuronal phosphatases through its PDZ domain and exerts its autocrine function in the brain with a distinct role in neuropoiesis (Kurschner and Yuzaki, 1999; Schwab et al., 2001; Bannert et al., 2003). Chemokine IL8 and pro-inflammatory cytokine IL18 are known to induce inflammatory mediator interferon γ and have been found to play neuroprotective roles in models of neurodegenerative conditions (Ryu et al., 2010; Semple et al., 2010).

Only a handful of recent studies have evaluated gene-specific DNA methylation and PTSD (Uddin et al., 2010, 2011; Ressler et al., 2011; Trollope et al., 2012) to our knowledge, and these studies have used only post-PTE blood samples. A cross sectional study of PTSD-affected and -unaffected individuals enrolled in a longitudinal study which investigated methylation and immune function profiles in DNA derived from whole blood in a methylation microarray reported that immune system functions were significantly overrepresented among the genes uniquely unmethylated in those with PTSD (Uddin et al., 2010). This signature included IL8, a gene which regulates innate and adaptive immune system processes (Uddin et al., 2010). There is emerging evidence that chemokines like IL8 and its receptors not only regulate immune cell infiltration but also contribute to a variety of physiological functions underlying neurotransmission, neuro-protection, and neurogenesis (Rostene et al., 2011) and therefore may exert a neuro-modulatory role by changing the balance of neurotransmitters and affecting fear memory (Herry et al., 2008; Joels et al., 2008). However, a peripheral blood mononuclear cell (PBMC) gene expression profile carried out in trauma survivors found that IL8 was under-expressed in PTSD cases, which would indicate the potential for hypermethylation of IL8 in these cases (Segman et al., 2005). Another recent human study found that persons who experienced traumatic events were at increased risk for PTSD, but only those with lower methylation levels of a serotonin transporter gene, SLC6A4. At higher methylation levels, individuals with more traumatic events were protected from PTSD (Koenen et al., 2011). Our study did not find a post-deployment case-control difference in this gene. We did find a pre-deployment case-control difference

in one position in IL8 (position 2) in that people who later became cases (pre-deployment cases) had lower levels of IL8 than did controls. This could indicate susceptibility to a PTE or to the development of PTSD, however we found it in only one position in the promoter region.

Serum has not previously been evaluated as a biomarker for DNA methylation patterns in cytokine gene promoter regions associated with PTSD. Most of the human studies which have investigated DNA methylation in PTSD have utilized predominantly whole blood-derived DNA (Uddin et al., 2010, 2011; Koenen et al., 2011; Ressler et al., 2011; Trollope et al., 2012). How correlated DNA methylation levels are in serum and whole blood to brain and other CNS tissues is not clear. However, it has been reported that some cytokines, such as IL18 can cross the blood-brain barrier (Alboni et al., 2010; Jung et al., 2012). The origin of circulating nucleic acids such as cell-free circulating DNA (cfcDNA) has been hypothesized to stem from necrosis or apoptosis (Gormally et al., 2007) though others report the possibility of an active release from cells (Anker et al., 1999; Stroun et al., 2001; Goebel et al., 2005; Rhodes et al., 2006). Excessive stress may induce DNA damage in the form of oxidized nucleosides, strand breaks, apoptosis, and necrosis and may be a source of cfcDNA in serum (Atamaniuk et al., 2011). Additionally, in the CNS, severe life stress leads to oxidative stress, and the accumulation of reactive oxygen species (ROS) is known to increase the susceptibility of brain tissue to damage (Schiavone et al., 2013). ROS, in turn, may also trigger molecular cascades leading to the blood-brain barrier permeability and neuronal death (Gu et al., 2011). Compared with cultured cells, clinical specimens, such as whole blood, serum, and even brain tissue and other CNS tissues, contain a heterogeneous mixture of cell types, each contributing its own unique methylation profile to the final analysis. Indeed DNA methylation patterns have been found to differ globally or locally, depending on the brain region/sub-region of focus (Iwamoto and Kato, 2009). One study demonstrated that the human cerebral cortex has an epigenetic signature distinct from the cerebellum (Ladd-Acosta et al., 2007) while others have shown that global DNA methylation differs across sub-regions of the rat hippocampus (Miller and Sweatt, 2007; Brown et al., 2008). These results taken collectively indicate that even different cortical cells are likely to have distinct epigenomic patterns. Serum DNA methylation patterns may provide a surrogate indicator of differential response to stress and PTSD, and our intent was to evaluate it as a non-invasive biomarker without drawing links to other types of tissues or directly extrapolating its significance. We evaluated difference in storage time of serum as a potential factor which may have influenced DNA methylation patterns. All samples for this study were obtained from the DoD Serum Repository in June 2009, and DNA was extracted from them soon thereafter. The time frame of serum being collected from our study subjects, based on the study sample criteria, was approximately January 2003 through June 2007. We found that cases and controls did not differ, either pre-deployment or post-deployment with respect to storage time. The mean storage time for cases pre-deployment was 4.5 years, for controls it was 4.8 years ($p = 0.15$). The mean storage time for cases post-deployment was 3.3 years, for controls it was 3.7 years ($p = 0.14$). As we expected, the storage time did differ by pre- and post-deployment time period. Thus for cases pre-deployment the mean was 4.5 years, while post-deployment it was 3.4 years ($p = < 0.01$). Likewise, the mean storage time for controls pre-deployment was 4.9 years, while post-deployment it was 3.7 ($p < 0.01$). Based on these differences, we adjusted all our models in which we carried out a pre/post comparison (i.e., **Table 2**; Table S1 in Supplementary Material) by storage time. However, after adjusting our models for difference in storage time between pre- and post-deployment samples (for both cases and controls), there were no changes in our adjusted means and *p*-values, so we opted for a more parsimonious model which did not include this variable.

Limitations of this study include lack of detailed information on deployment exposures for both the cases and controls. Deployment was used as a proxy for a PTE, and the exact timing of a PTE, if it occurred, is not known. There is also the issue that cases, by virtue of their PTSD diagnosis, were presumably exposed to a PTE, while we do not know the same for controls. In an attempt to investigate a possible bias from this disparity, we re-ran all our analyses utilizing a control group comprised only of those likely involved in combat during deployment (the group specified in **Table 1** as "more likely involved in combat"). The methylation patterns we found did not differ from those using the complete control group (data not shown). We also tried to control for potential differences in deployment experience by ensuring that all cases and controls had not been previously deployed, that they were all active duty Army or Marines, and that they were deployed from 6 to 18 months, but there is still potential for significant variation among all our subjects with respect to intensity of combat during deployment. We do not have any data on previous exposure to a PTE or underlying personality traits, which have been hypothesized to influence vulnerability to PTSD (van Zuiden et al., 2011). Military personnel, prior to deployment, undergo a medical exam and are required to complete a pre-deployment health assessment survey (Department of Defense form 2795) to ensure medical readiness for deployment. This would have applied to all subjects in our study.

The timing of sample collection was not standardized, so there was heterogeneity in the length of time between deployment and serum draw. Although we tried to minimize this interval, the timing of each serum sample being added to the DoDSR depended on the timing of HIV testing for each military service member. We do not have data on other relevant exposures which may influence DNA methylation, such as dietary factors (folate, vitamin B_{12} intake) (Fenech, 2001; Piyathilake and Johanning, 2002) smoking (Toh et al., 2010), and alcohol consumption (Seitz and Stickel, 2007; Toh et al., 2010; Zhu et al., 2012).

Ascertainment of PTSD via query of ICD-9 coded medical encounter is not ideal. Although we attempted to restrict the definition of PTSD to a scenario which would minimize misclassification of disease, by requiring the ICD-9 code 309.81 be present in the first diagnostic position for two outpatient records spaced at a reasonable calendar time distance, this type of case ascertainment is still prone to misclassification. However, this is the PTSD case definition developed in September 2008 by the Department of Defense Interagency PTSD and TBI Standardization Committee, and it has been accepted by Military Health Affairs for surveillance

(Armed Forces Health Surveillance Center, 2011). The DNA yields from the sera in this study were small, another potential limitation.

The limitations mentioned above are offset by the rare access to both pre- and post-deployment biologic samples in cases and controls. While most case-control studies would not be able to infer whether the observed methylation patterns were a consequence of PTSD or whether they indicated vulnerabilities that existed among the cases before the onset of PTSD, our study was able to address both possibilities. Another strength of the study is that we utilized pyrosequencing to provide a quantitative measure of DNA methylation. Pyrosequencing is a quantitative real-time sequencing technology. The light output or the Pyrosequencing signals (represent as Relative Light Unit or RLU) are directly proportional to the number of molecules being sequenced. Pyrosequencing QCpG software has a number of quality assessments to pass or to fail results for each sample being sequenced. These specifications give information including sample quality, non-specific PCR amplification, non-specific sequencing, and completion of bisulfite modification. Therefore, if a pyrosequencing run on a specific sample gave a passing result, it indicates that the DNA of that sample is of sufficient quality.

Chance could account for some of the statistically significant findings we report, given the number of comparisons and the small sample size, but this was an exploratory study and the results should be considered as preliminary. The DoDSR, which contains over 45 million longitudinally collected serum samples of U.S. military service members will provide a vast resource for carrying out future investigations in larger populations. There is growing evidence that the molecular mechanisms that regulate DNA methylation are involved in synaptic plasticity, learning, and memory (Sananbenesi and Fischer, 2009). DNA is inherently stable, compared with RNA, thus development of biomarkers based on DNA provide an attractive potential. Additionally, since modifications in DNA methylation can potentially be reversed – by de-methylating or methylating agents – understanding the role of DNA methylation in PTSD has the potential to fuel novel approaches to PTSD treatment.

CONCLUSION

We evaluated DNA methylation from serum as a potential biomarker for PTSD. In this exploratory study of deployed personnel, comparing those with PTSD and those who did not develop PTSD, the latter had reduced %5-mC levels of H19 and IL18 after deployment and elevated levels of IL18 after deployment. Additionally, pre-deployment the people who later became cases had lower levels of IL18 %5-mC compared with controls. Whether these are markers of vulnerability or resilience is a matter of speculation, but because of the high incidence of PTSD, it is important to study these patterns further.

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DISCLAIMERS

The views expressed in this scientific paper are those of the author(s) and do not reflect the official policy or position of the U.S. government or the Department of Defense.

AUTHORS CONTRIBUTION

Jennifer A. Rusiecki was the PI of the study and conceived the original idea, designed the study, managed the study, and directed the data analysis and interpretation. Celia Byrne was involved in the interpretation of the study pertaining to epidemiologic factors. Zygmunt Galdzicki was involved in the interpretation of the study pertaining to cytokines and other genes. Vasantha Srikantan was involved in the study design and initial lab preparation of the samples. Ligong Chen carried out all statistical analyses. Matthew Poulin carried out the measurement of methylation and was involved in the interpretation of the results. Liying Yan carried out the measurement of methylation and was involved in the interpretation of the results. Andrea Baccarelli was involved in the original study design and provided expertise throughout the entire project and was involved in the interpretation of the findings. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Molecular_Psychiatry/10.3389/fpsyt.2013.00056/abstract

Table S1 | Generalized linear models for investigating the adjusted means* and differences between controls pre- and post-deployment and cases pre- and post-deployment.

Table S2 | Generalized linear models investigating the adjusted means* and differences between cases and controls, pre-deployment and post-deployment.

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Epigenetic biomarkers as predictors and correlates of symptom improvement following psychotherapy in combat veterans with PTSD

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Epigenetic alterations offer promise as diagnostic or prognostic markers, but it is not known whether these measures associate with, or predict, clinical state. These questions were addressed in a pilot study with combat veterans with PTSD to determine whether cytosine methylation in promoter regions of the glucocorticoid related *NR3C1* and *FKBP51* genes would predict or associate with treatment outcome. Veterans with PTSD received prolonged exposure (PE) psychotherapy, yielding responders ($n = 8$), defined by no longer meeting diagnostic criteria for PTSD, and non-responders ($n = 8$). Blood samples were obtained at pre-treatment, after 12 weeks of psychotherapy (post-treatment), and after a 3-month follow-up. Methylation was examined in DNA extracted from lymphocytes. Measures reflecting glucocorticoid receptor (GR) activity were also obtained (i.e., plasma and 24 h-urinary cortisol, plasma ACTH, lymphocyte lysozyme $IC_{50\text{-}DEX}$, and plasma neuropeptide-Y). Methylation of the GR gene (*NR3C1)* exon 1F promoter assessed at pre-treatment predicted treatment outcome, but was not significantly altered in responders or non-responders at post-treatment or follow-up. In contrast, methylation of the FKBP5 gene (*FKBP51)* exon 1 promoter region did not predict treatment response, but decreased in association with recovery. In a subset, a corresponding group difference in FKBP5 gene expression was observed, with responders showing higher gene expression at post-treatment than non-responders. Endocrine markers were also associated with the epigenetic markers. These preliminary observations require replication and validation. However, the results support research indicating that some glucocorticoid related genes are subject to environmental regulation throughout life. Moreover, psychotherapy constitutes a form of "environmental regulation" that may alter epigenetic state. Finally, the results further suggest that different genes may be associated with prognosis and symptom state, respectively.

Keywords: PTSD, veterans, epigenetics, methylation, promoter, glucocorticoid receptor, FK506 binding protein 5, psychotherapy

INTRODUCTION

Cytosine methylation of glucocorticoid related genes represents an epigenetic modification thought to underlie the developmental programing of hypothalamic-pituitary-adrenal (HPA) axis function (1). Plasticity of the epigenome appears to constitute a molecular mechanism whereby genetic predispositions may be influenced by environmental exposures resulting in sustained alterations in gene expression and protein synthesis (2–4). Epigenetic modifications of a glucocorticoid receptor (GR) gene promoter were first described in the rat as a mechanism by which variations in parent – offspring interactions influence HPA-axis and behavioral responses to stress (5, 6). Maternal care regulates the methylation state of the GR exon 1_7 promoter in hippocampus, which in turn, regulates GR expression, the capacity for glucocorticoid negative feedback, and HPA-axis responses to stress (5, 7). Subsequent studies in humans showed that childhood adversity associates with higher methylation of the GR exon 1F promoter (the human ortholog of the rat exon 1_7 promoter sequence) lower hippocampal GR expression and increased HPA-axis responses to stress (8, 9).

Recent studies reveal additional mechanisms for the influence of childhood adversity on GR signaling and HPA-axis function.

FK506 binding protein 5 (FKBP5) regulates intracellular GR signaling by decreasing ligand binding and restricting GR translocation to the nucleus (10, 11). GR activation induces *FKBP51* (the FKBP5 gene) transcription, thus establishing an intracellular feedback loop that moderates GR sensitivity (12). FKBP5 genetic variants in interaction with childhood adversity predict the risk for affective disorders, including major depression, suicide attempts, and PTSD (13–16). Moreover, the methylation state of selected CpGs across the *FKBP51* gene is determined by an interaction between sequence-polymorphism and childhood adversity, and modulates sensitivity of FKBP5 to GR regulation (17). Various aspects of the GR (*NR3*C1) and FKBP5 genes, including genotype and gene expression, have been implicated in PTSD (12, 13, 15, 18–27). Low FKBP5 gene expression in PTSD has been associated with low plasma cortisol and PTSD severity (21, 24). Taken together, these findings suggest that childhood adversity influences the epigenetic state and transcriptional activity of genes that regulate HPA-axis responses to stress.

Importantly, stress reactivity predicts the risk for multiple affective disorders, as well as PTSD (28). Early adverse experiences are risk factors for PTSD following adult trauma exposures (29–31); thus the associated epigenetic states may represent a molecular mechanism responsible for altering subsequent responses to environmental adversity (4, 32). Neuroendocrine studies reveal that the development of PTSD following trauma exposure is associated with pre-traumatic biological markers that reflect prior sensitization to stress (33). Relatively stable changes in methylation potentially explain the chronicity and tenacity of symptoms observed in PTSD. In PTSD there is neither a complete restoration of baseline hormone levels following trauma, nor do persons with this condition feel that they have returned to a pre-trauma psychological state. PTSD is a condition that has been associated with low glucocorticoid levels, enhanced GR sensitivity, and insufficient glucocorticoid signaling (34–37). Epigenetic signals associated with childhood adversity offer a potential explanation both for why stress responses do not abate once an immediate threat is no longer present, as in the case of PTSD, and for the fact that some persons are at greater risk than others for the development of PTSD (32, 38). In fact, many of the alterations noted in PTSD have been demonstrated in association with early adversity regardless of the subsequent development of PTSD in adults (9, 17, 39, 40). On the other hand, persons who develop PTSD can also recover from this condition either spontaneously or in response to treatment (41). Moreover, an emerging trajectory in PTSD is one in which there are fluctuating symptoms, which maybe mediated by external post-traumatic environmental circumstances. This raises the possibility that some epigenetic changes, originally induced by the environment, change over time in response to subsequent challenges.

The goal of the current study was to examine methylation of the GR and FKBP5 genes – and associated downstream neuroendocrine measures, cortisol, and NPY, before and after prolonged exposure (PE) psychotherapy in veterans with PTSD. The exon 1F promoter was selected as the most biologically relevant GR promoter region for methylation analysis because this region corresponds to exon 1_7 of the rat GR gene, shown to be differentially methylated in the rat hippocampus based on variations in maternal care (5), and in human peripheral blood and hippocampal post-mortem tissue in association with child abuse (9, 39, 40). We hypothesized that higher GR exon 1F promoter methylation would predict treatment response and "normalization" of PTSD related biology at post-treatment time-points but would not itself change appreciably over time. We also examined the FKBP5 exon 1 promoter methylation and, based on previously observed changes in FKBP5 gene expression in association with PTSD symptom severity (24), we hypothesized that *FKBP5* promoter methylation would change in responders, in association with glucocorticoid related measures.

The examination of biological measures in association with PTSD symptom change following an efficacious psychotherapy trial was designed to yield a sample with a variable degree of symptom improvement, with some showing large decreases in symptom severity, and others, minimal or moderate change. An additional advantage of this approach is the ability to modify symptoms without introducing exogenous medications that might have direct effects on the biological measures of interest. The participants for this study were drawn from a larger pool of combat veterans that were examined as part of an effort to identify neuroendocrine markers (e.g., cortisol, NPY) that would distinguish diagnostic, state related, and recovery markers in combat veterans randomized to PE or a minimal attention (MA) condition. To accomplish the larger objective, combat veterans were assessed for blood and urinary biomarkers prior to, and after completing, 12 weeks of treatment – either PE or MA – and after a 3-month naturalistic follow-up (for those who received PE). The direct manipulation of target symptoms with psychotherapy within a relatively short period of time (weeks to months) permits identification of biomarkers associated with relatively rapid symptom change and treatment-associated recovery. Assessment prior to and following psychotherapy allows differentiation of prognostic indicators from state markers of symptom change. Markers that do not change as symptoms improve may be prognostic indicators or reflect measures associated with risk for PTSD. Previous results from a preliminary study of combat veterans demonstrated that GR responsiveness predicted treatment outcome (42). Because it was of interest to draw specific conclusions about symptom change in association with a structured psychotherapy, in this report we only include participants in the active arm (i.e., who received and completed PE) who completed the pre-treatment, post-treatment, and follow-up assessments.

MATERIALS AND METHODS

PARTICIPANTS

This report represents a subsample ($n = 16$) of a larger study of 113 combat veterans who enrolled in a clinical trial comparing the effects of PE to a MA condition, conducted at the James J. Peters Bronx VA Medical Center (JJP BVAMC). Results of the subset of completers will be reported elsewhere. The current subsample comprised 14 men and 2 women who completed PE treatment. Nine were Vietnam veterans, and seven had recently returned from active duty in Iraq or Afghanistan. The decision to study PE completers in this subset was based on two considerations. First, following MA, the participants were allowed to begin active psychotherapy. For this reason, the initial study did not have a

follow-up evaluation for those receiving MA. Second, by comparing participants who received the same intervention, biological correlates of symptom severity are not confounded with effects of treatment type.

As the molecular measures reported here were not part of the original protocol, selection of this subgroup was based on (1) having agreed to the future use of their biological samples; (2) having completed all three evaluations (pre-treatment, post-treatment, and follow-up); (3) having participated in the PE condition; and (4) having sufficient remaining sample for the analysis of promoter methylation of GR and FKBP5 genes after other study measures had been obtained. Participants in this subsample were not appreciably different from those who completed PE in the parent study with respect to pre-treatment demographic or clinical variables, or post-treatment measures. All procedures were approved by the IRB at the JJP BVAMC, all participants signed written, informed consent prior to initiation of study procedures.

Inclusion/exclusion criteria

Following a comprehensive medical (including lab testing) and psychological evaluation, participants were excluded if they did not experience a Criterion A traumatic event during military service or meet DSM-IV criteria for current PTSD with a duration of at least 6 months. Additional exclusion criteria included having significant illness that would interfere with interpretation of biological data, such as insulin-dependent diabetes, seizure disorder, or any disease requiring ongoing treatment with systemic steroids; regular use of benzodiazepines or oral steroids; a BMI >40; smoking more than two packs per day; meeting criteria for substance abuse or dependence within the last 6 months; a lifetime history of schizophrenia, schizoaffective disorder, bipolar disorder, obsessive compulsive disorder, or being in any acute clinical state that necessitated prompt initiation of pharmacotherapy or other treatment, including assessed suicide risk. Veterans receiving psychotropic medications for PTSD were eligible to participate if they had maintained a stabilized therapeutic dose for a minimum of 2 months prior to randomization.

PROCEDURE

A comprehensive psychological evaluation was performed by a clinical psychologist at the three study time-points (pre- and post-treatment, follow-up). Several structured diagnostic instruments were used including the Structured Clinical Interview for DSM-IV (SCID) (43), and the Clinician Administered PTSD Scale (CAPS) (44). The CAPS additionally provided a continuous measure of symptom severity of PTSD. The PTSD Symptom Scale – Self-Report Version (PSS-SR) was used as a self-report of PTSD symptoms (45). Two self-report measures were administered to assess childhood trauma and life events. The Childhood Trauma Questionnaire (CTQ) was used to assess early trauma (46), and the Deployment Risk and Resiliency Inventory (DRRI) to access military and civilian life events pre- and post-deployment (47). For all subjects, an independent evaluator (i.e., not the individual who provided treatment) assessed clinical outcome following treatment.

Biological measures

The primary molecular measures included GR- and FKBP5-promoter methylation. These were obtained in parallel with the psychological assessments. We also examined FKBP5 gene expression in subjects for whom there was sufficient sample. A battery of HPA-axis markers was examined as part of the parent study to assess basal cortisol levels and GR responsiveness. Reported here are the biological measures that should be functionally related to the molecular measures and/or may vary in response to symptom change. These include basal plasma cortisol, 24 h-urinary cortisol levels, plasma ACTH, and cortisol responses to a low dose (0.50 mg) dexamethasone suppression test (DST), glucocorticoid sensitivity as assessed by the lymphocyte lysozyme $IC_{50\text{-}DEX}$, and plasma NPY.

Sample processing and hormone determination

Blood samples pre- and post-dexamethasone were collected as previously described (48). Plasma was extracted from EDTA containing tubes, aliquoted, and frozen at −80°C until subsequent hormonal analysis. Urine samples were collected over a 24-h period as previously described (49). Cortisol (plasma and urinary), dexamethasone, and NPY were determined by radioimmunoassay as previously described (48, 50). Plasma ACTH was determined using an enzyme-linked immunosorbent assay (ELISA; ALPCO Diagnostics, Salem, NH, USA). The intra- and inter-assay coefficients of variation were 4.7 and 7.1% for ACTH, 2.3 and 6.1% for cortisol, 8.0 and 9.0% for dexamethasone, and 3.5 and 11.6% for NPY, respectively.

Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were purified from basal EDTA pretreated blood by Ficoll-Paque (Amersham, UK) using Accuspin tubes (Sigma-Aldrich, Saint Louis, MO, USA). After two washes in Hanks' Balanced Salt Solution (Life Technologies, Grand Island, NY, USA), PBMCs were counted with a hemocytometer. Some cell pellets were immediately used for determination of lysozyme $IC_{50\text{-}DEX}$ as previously described (51). Some cell pellets were quickly frozen, stored at −80°C, and later used for DNA extraction (see below) and a portion of cell pellets was dissolved in TRIzol Reagent (Invitrogen, CA, USA) by adding 1 ml of the reagent per 1×10^7 cells, quickly frozen, stored at −80°C, and later used for RNA extraction (see below).

DNA cytosine methylation sodium bisulfite mapping

Genomic DNA was extracted from frozen PBMC pellets following the Flexigene DNA kit protocol (Qiagen, Valencia, CA, USA). Methylation mapping of the human GR exon 1F promoter (**Figure 1A**) was performed following Dr. Meaney's laboratory recommendations and as previously described for human hippocampus (9). The methylation mapping method for the human FKBP5 proximal promoter located upstream of exon 1 (**Figure 1B**) was developed in Dr. Yehuda's laboratory. Sodium bisulfite treatment was carried out according to the EpiTect Bisulfite kit protocol (Qiagen, Valencia, CA, USA). In each sodium bisulfite conversion reaction, 0.8 μg of genomic DNA was used. In the same experiment, 0.8 μg of Universal Methylated Standard (Zymo Research, Irvine, CA, USA) was treated

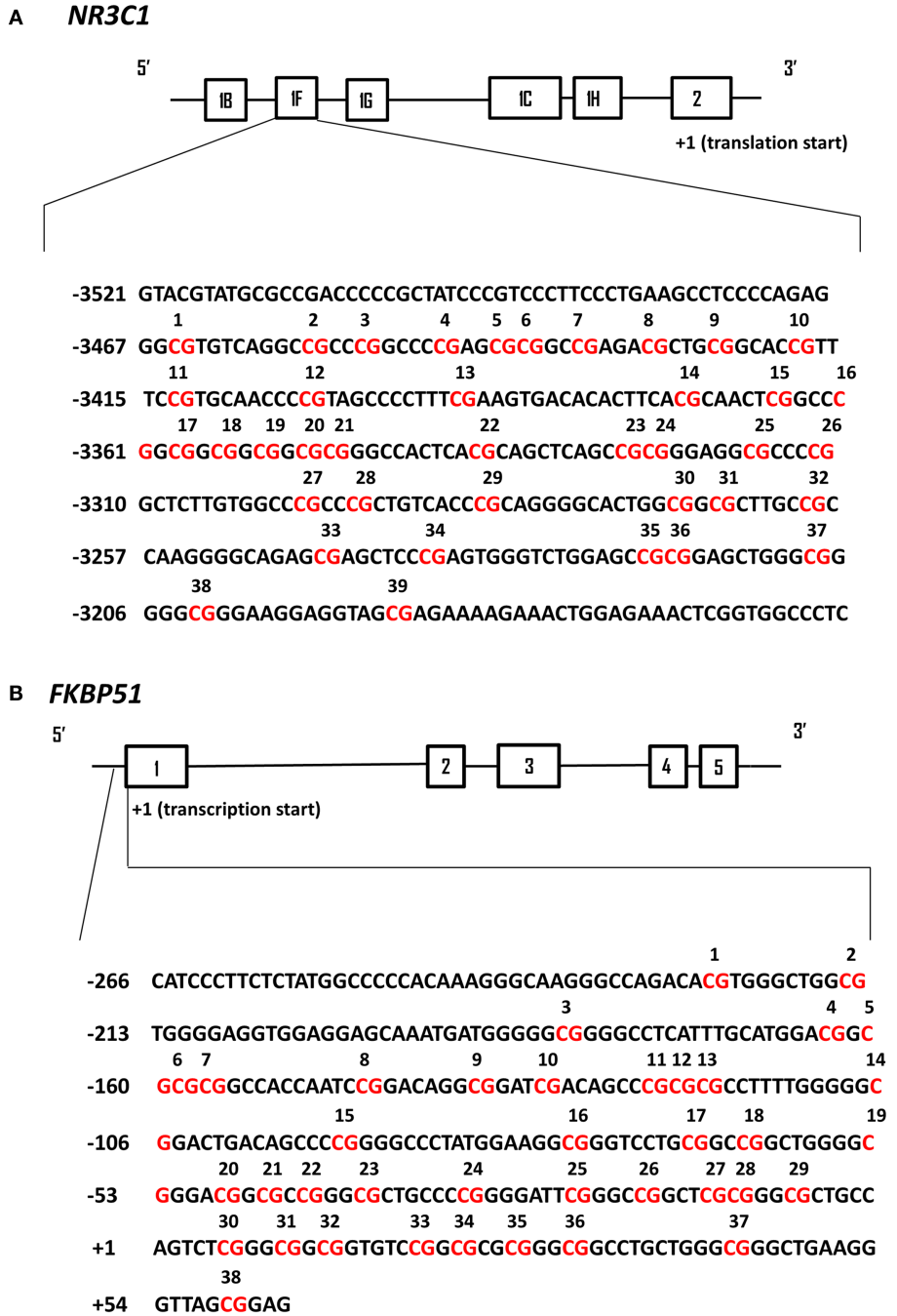

FIGURE 1 | Schematic representation of human *NR3C1* and *FKBP51* exon 1 promoter regions analyzed by DNA cytosine methylation bisulfite mapping. In both panels the solid black line boxes with a number represent the different exons and the 5′–3′ orientation goes from left to right. **(A)** The *NR3C1* gene 5′ region is composed of multiple first exons and the translation start site is located within exon 2. The numbering of exon 1F promoter is based on the translational start site (+1). The CpG sites that have been analyzed by bisulfite sequencing are in red and numbered. **(B)** The *FKBP51* gene proximal promoter region is numbered based on the transcriptional start site (+1) of exon 1. The CpG sites that have been analyzed by bisulfite sequencing are in red and numbered.

with sodium bisulfite to check completion of the sodium bisulfite reaction. The genomic region of the human GR exon 1F promoter was subjected to PCR amplification using the following primer sequences: 5′-GTG GTG GGGGAT TTG-3′ (forward); 5′-ACCTAATCTCTCTAAAAC-3′ (reverse) following previously published procedures (9). The thermocycler protocol involved an initial denaturation (5 min, 95°C), 35 cycles of denaturation (1 min, 95°C), annealing (2 min 30 s, 55°C),

and extension (1 min, 72°C), and then a final extension (5 min, 72°C) with subsequent cooling at 4°C. The resulting PCR product was subjected to another round of PCR, using the following nested primers: 5′-TTTTTGAAGTTTTTTTAGAGGG-3′ (forward); 5′-AATTTCTCCAATTTCTTTTCTC-3′ (reverse). The thermocycler protocol was the same as the initial PCR procedure except that the extension step was prolonged to 10 min. The genomic region of the human FKBP5 exon 1 promoter was subjected to PCR amplification using the following primer sequences: 5′-GGTAGGTTTTGTGGATAGATAGGA-3′ (forward); 5′-ACTCCGCTAACCCTTCAAC-3′ (reverse). The thermocycler protocol involved an initial denaturation (4 min, 95°C), 35 cycles of denaturation (30 s, 95°C), annealing (30 s, 45°C), and extension (1 min, 72°C), and then a final extension (10 min, 72°C) with subsequent cooling at 4°C. The resulting PCR product was subjected to another round of PCR, using the following nested primers: 5′-AGGGGGTGTTAGTTTTTATTATTTTTT-3′ (forward); 5′-ACTCCGCTAACCCTTCAAC-3′ (reverse). The thermocycler protocol was the same as the initial PCR procedure. The resulting PCR products were analyzed on a 2% agarose gel and then purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The PCR products were subcloned using a PCR product cloning kit (Qiagen) and individual plasmid containing the ligated promoter regions were extracted and sequenced (Genewiz, Inc., South Plainfield, NJ, USA). The sequences for 20 individual clones were aligned and analyzed in the DNA Alignment software program BioEdit (Ibis Biosciences, Carlsbad, CA, USA). The DNA samples were analyzed in batches of 20–30 samples. Variability in the DNA bisulfite treatment did not exceed 2% between the batches.

Gene expression

RNA, from Trizol-dissolved PBMCs, was extracted using commercially available kits (RNeasy Mini Kit and RNeasy MinElute Cleanup Kit, Qiagen). Extracted RNA was evaluated for its quality using NanoDrop 2000 Spectrophotometer (Thermo Scientific). RNA was aliquoted and stored at −80°C until use.

For GR exon 1F expression, cDNA synthesis was completed using Maxima reverse transcriptase (Thermoscientific) and GR target oligo (CAG GGG TGC AGA GTT CGA TG) since GR expression levels are very low in blood cells. Quantitative real-time PCR was performed with a LightCycler 480 (Roche Applied Science). *NR3C1* exon 1F primers (forward primer 5′-AAG AAA CTG GAG AAA CTC GGT GGC-3′, reverse primer 5′-TGA GGG TGA AGA CGC AGA AAC CTT-3′) and RT^2 PCR primer sets for two endogenous reference genes (β2 microglobulin, Catalog no. PPH01094E; SABiosciences and glyceraldehyde-3-phosphate dehydrogenase, Cat#PPH14985F, Sabioscience) were used. Only one cDNA was amplified in each PCR (monoplex).

For FKBP5 expression, cDNA was synthesized by reverse transcription reaction using High capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed using an ABI Step One Plus Real-Time PCR Instrument (Applied Biosystems) and TaqMan probes (Applied Biosystems). The primers used to target exon junctions 7–8 and 8–9 of the *FKBP51* gene, and four endogenous reference genes, have been previously described (21). Only one cDNA was amplified in each PCR (monoplex).

The reactions were run in triplicate for each sample and were quantitated by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle, Ct). To account for the differences in the amounts of input material across samples, the expression level of each transcript in each sample was normalized to the geometric mean of the expression levels of the endogenous reference genes using the $2^{-\Delta\Delta Ct}$ method.

STATISTICAL ANALYSIS

Responder status was defined by the presence or absence of PTSD at post-treatment evaluation, as determined by an independent psychologist using the CAPS for DSM-IV. For GR and FKBP5, number of methylated sites in the CpG region examined for each subject was calculated by observing the percentage of methylated clones at each site and then totaling the number of sites with percentages greater than zero. Because the number of individual sites examined was 39 for GR and 38 for FKPB5, the potential range of number of methylated sites for GR is 0–39, with an actual range of 1–16; the potential range for FKBP5 is 0–38, with an actual range of 2–20. An alternative measure for promoter methylation was the sum % methylation. For this measure, at each site of the promoter region, the total number of methylated clones (out of 30) was converted to a percentage. The percentages across all sites were then added to create a total summed percentage of methylation.

Measures of central tendency and variability (mean and SE) were calculated at baseline, treatment completion, and follow-up for all continuous primary and secondary clinical outcome measures and biological variables. Baseline comparisons of group differences were conducted using independent samples *t*-tests for continuous variables and chi-square analysis for categorical variables. Correlation analyses were conducted to determine appropriate covariates for repeated measures analysis. Repeated measures ANOVAs and ANCOVAs were conducted using responders and non-responders to explore within and between group changes on biological and psychological measures in order to determine predictors and correlates of treatment outcome. Additional bivariate correlations were used to measure association of GR- and FKBP5-promoter methylation at pre-treatment with clinical and other biological variables at post-treatment or follow-up and of post-treatment variables with those at follow-up. For the correlational analysis, the number of methylated sites was selected as the most sensitive measure of methylation in this study. Statistical significance for all analyses was set at $p < 0.05$.

RESULTS

DEMOGRAPHIC, DESCRIPTIVE, AND CLINICAL MEASURES

Table 1 reports comparisons of the responder and non-responder groups at baseline on a variety of demographic and descriptive characteristics. There were significant group differences in age indicating that responders tended to be younger, had PTSD for a shorter duration, and had fewer total lifetime traumatic events, than non-responders. A chi-square analysis of the number of veterans in the two conflicts in relation to responder status did not reach statistical significance in this small sample. Moreover, initial PTSD symptom severity was comparable for responders and non-responders, as assessed by clinician or self-report (**Table 2**).

Table 1 | Baseline characteristics comparing responders to non-responders.

	Responders ($n=8$) M (SD) or %	Non-responders ($n=8$) M (SD) or %		
Age	41.25 (17.82)	57.88 (7.45)	$t_{(9.4)}{}^{d} = -2.435$	$p = 0.037$
Years of education	13.38 (2.20)	15.50 (2.39)	$t_{(14)} = -1.850$	ns
Marital status	Single (37.5%) Married or living with partner (62.5%)	Single (37.5%) Married or living with partner (62.5%)	$\chi^2_{(1)} = 1.000$	ns
Ethnicity	Hispanic (37.5%) Black (37.5%) White (25%)	Hispanic (50%) Black (37.5%) White (12.5%)	$\chi^2_{(2)} = 0.476$	ns
Conflict	OEF/OIF (62.5%) Vietnam (37.5%)	OEF/OIF (25%) Vietnam (75%)	$\chi^2_{(1)} = 2.286$	ns
Stabilized on psychotropics	Yes (62.5%)	Yes (62.5%)	$\chi^2_{(1)} = 1.000$	ns
Lifetime CAPS[a] total score	92.75 (12.70)	105.25 (15.15)	$t_{(14)} = -1.789$	ns
CTQ[b] total	9.43 (3.72)	10.81 (4.02)	$t_{(14)} = -0.717$	ns
Time since first DRRI[c] trauma (years)	25.00 (17.85)	45.00 (8.83)	$t_{(10.2)}{}^{d} = 2.841$	$p = 0.017$
DRRI pre-deployment life events	4.50 (3.16)	7.25 (3.15)	$t_{(14)} = -1.742$	ns
DRRI post-deployment life events	5.13 (3.68)	10.38 (2.56)	$t_{(14)} = -3.312$	$p = 0.005$
DRRI total life events	9.63 (5.80)	17.63 (3.16)	$t_{(14)} = -3.424$	$p = 0.004$

[a] *Clinician Administered PTSD Scale;*

[b] *Childhood Trauma Questionnaire;*

[c] *Deployment Risk and Resilience Inventory;*

[d] *unequal variance t-test.*

Table 2 | Interview and self-report measures at before and after treatment and at 12-week follow-up in Responders (R) and Non-responders (NR).

	Pre-treatment		Post-treatment		Follow-up		*p*-Value		
	R	NR	R	NR	R	NR	Group	Time	G × T
Current PTSD severity[a]	75.00 (6.39)	81.75 (6.39)	26.50 (6.78)	70.50 (6.78)	30.00 (7.72)	61.75 (7.72)	**0.004**	**0.000**	**0.003**
Self-rated PTSD severity[b]	36.50 (2.39)	35.17 (2.76)	18.75 (3.98)	34.50 (4.60)	14.63 (3.15)	32.00 (3.63)	**0.016**	**0.000**	**0.004**

[a] *Clinician Administered PTSD Scale (CAPS);*

[b] *PTSD Symptom Scale-Self-Report (PSS-SR).*

TREATMENT RESPONSE AND CLINICAL INDICATORS

Table 2 also describes changes in measures of PTSD. Consistent with defining groups on the basis of their diagnostic status at post-treatment, there were significant group × time interactions for PTSD symptom severity.

METHYLATION OF A GR PROMOTER

Levels of methylation across the GR exon 1F promoter were generally low, as expected of CpG sites lying within a CpG island. **Figure 2A** demonstrates a significant group difference in the number of CpG methylated sites across the GR exon 1F promoter between responders and non-responders at pre-treatment ($t_{14} = 2.43$, $p = 0.029$), with a greater average number of methylated sites in responders (4.5 ± 0.6) than non-responders (2.5 ± 0.6). A similar pre-treatment difference in GR exon 1F promoter methylation was observed when the sum % methylation measure (**Figure 2B**) was used ($t_{14} = 2.29$, $p = 0.045$; responders 28.7 ± 5.9, non-responders: 13.8 ± 2.8).

In repeated measures analysis of number of methylated sites, there was a main effect of group ($F_{1,14} = 7.584$, $p = 0.016$) across the three time-points, but no significant effect of time ($F_{2,28} = 2.41$, ns) or group × time interaction ($F_{2,28} = 0.171$, ns). The significant group effect reflects higher level of number of methylated CpG sites in samples from responders compared to non-responders. Similar effects at a trend level of significance were observed when using the sum % methylation measure (group: $F_{1,14} = 3.627$, $p = 0.078$, trend; time: $F_{2,28} = 2.22$, ns, group × time interaction: $F_{2,28} = 0.401$, ns).

Treatment response for individual subjects was predicted by pre-treatment GR exon 1F promoter methylation. Pre-treatment levels of GR exon 1F promoter methylation were significantly correlated with both post-treatment PTSD symptom severity (**Figure 3A**) and the change in symptom severity from pre- to post-treatment (**Figure 3B**). Higher post-treatment GR exon 1F promoter methylation also predicted lower self-reported (but not clinician-rated) PTSD symptoms at follow-up (**Figure 4**).

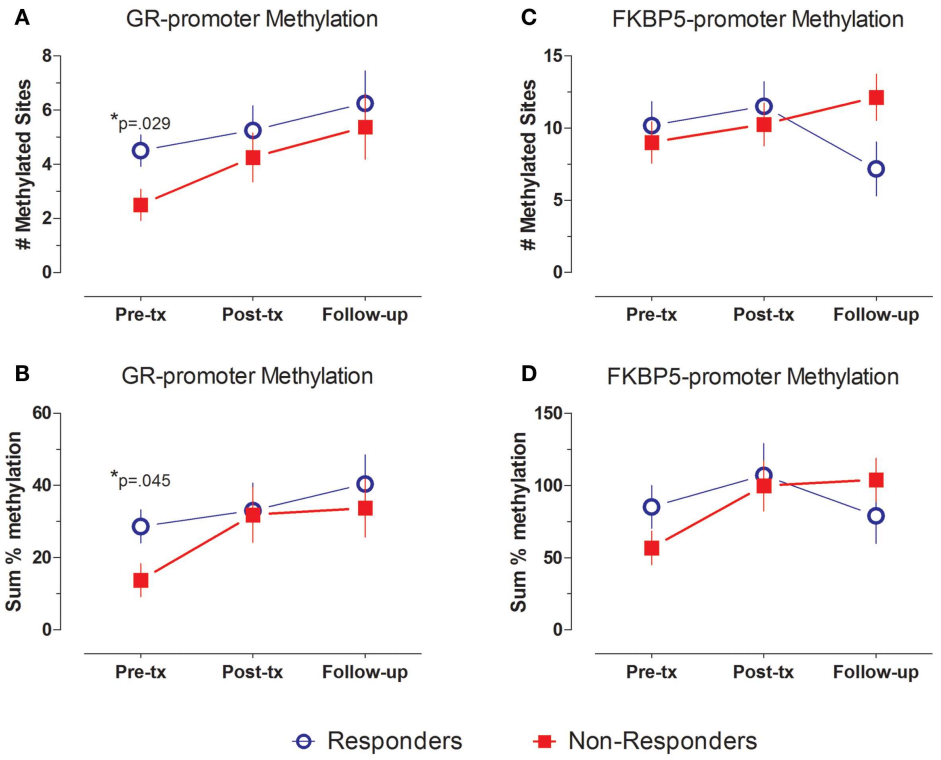

FIGURE 2 | GR- and FKBP5-promoter methylation at pre-treatment, post-treatment and follow-up. GR- **(A,B)** and FKBP5-promoter **(C,D)** methylation shown by mean ± SE of number of methylated sites **(A,C)** or sum % methylation **(B,D)**. Responders ($n = 8$) to treatment are represented by blue, open circles and non-responders ($n = 8$) by red squares. For GR exon 1F promoter methylation, there is a significant group difference at Pre-treatment, but no main effect of time and no group by time interaction. FKBP5 promoter methylation shows a significant group by time interaction, but no main effects of group or time. Statistical significance was set at $p < 0.05$.

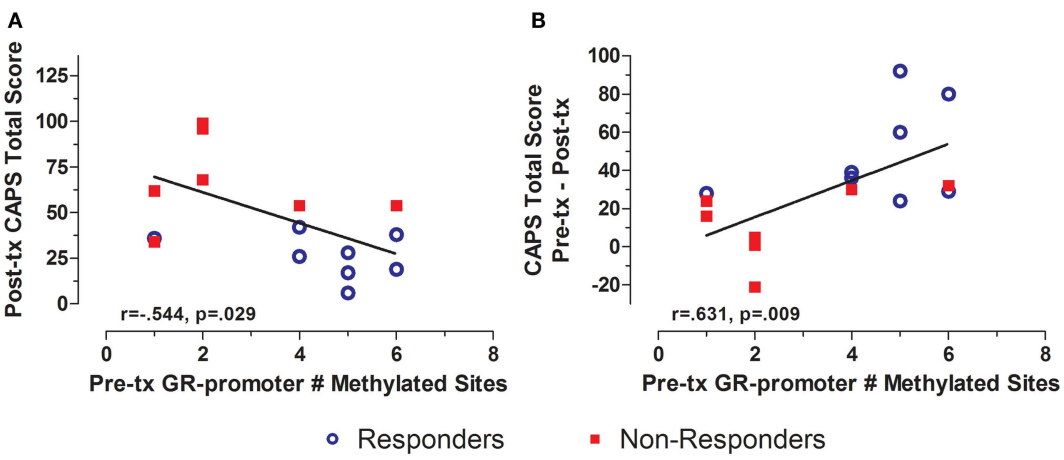

FIGURE 3 | Relationship between GR exon 1F promoter methylation at pre-treatment and PTSD symptom severity at post-treatment. Correlations of pre-treatment GR exon 1F promoter methylation (# of methylated sites) with post-treatment CAPS total score **(A)** and change in CAPS total score from pre- to post-treatment **(B)**. Responders ($n = 8$) to treatment are represented by blue, open circles and non-responders ($n = 8$) by red squares. The higher number of GR exon 1F promoter methylated sites at pre-treatment corresponded to a lower CAPS total score at Post-treatment and a greater reduction in symptoms from pre- to post-treatment. Correlation coefficients are denoted in the different panels. Statistical significance was set at $p < 0.05$.

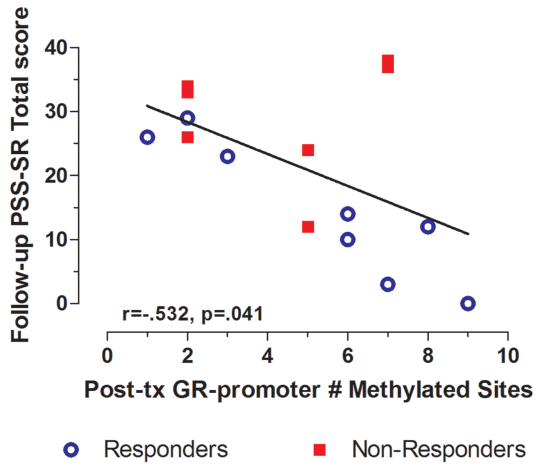

FIGURE 4 | Relationship between GR exon 1F promoter methylation at post-treatment and self-reported PTSD symptom severity at follow-up. Correlations of Post-treatment GR exon 1F promoter methylation (# of methylated sites) with PSS-SR total score at follow-up. Responders ($n = 8$) to treatment are represented by blue, open circles and non-responders ($n = 7$) by red squares. The higher number of GR exon 1F promoter methylated sites at post-treatment corresponded to a lower PSS-SR total score at follow-up ($r = -0.532$, $p = 0.041$). Statistical significance was set at $p < 0.05$.

Pre-treatment GR exon 1F promoter methylation additionally predicted several post-treatment biological measures. Pre-treatment GR exon 1F promoter methylation was positively associated with post-treatment 24 h-urinary cortisol levels (**Figure 5A**) and plasma NPY (**Figure 5B**). Although only at a trend level of significance, pre-treatment GR exon 1F promoter methylation was associated with follow-up glucocorticoid sensitivity as determined by the lymphocyte lysozyme test 3 months after treatment ended (**Figure 5C**). Note that lower $IC_{50\text{-}DEX}$ indicates greater glucocorticoid sensitivity. Importantly, there were no significant correlations observed cross-sectionally between GR exon 1F promoter methylation and PTSD symptoms or other endocrine measures in this sample.

METHYLATION OF THE FKBP5 PROMOTER

In contrast to the findings for the GR exon 1F promoter, FKBP5 promoter number of methylated sites showed variation in association with treatment outcome reflected in a significant group by time interaction effect ($F_{2,24} = 4.576$, $p = 0.021$). Responders showed a decrease, whereas non-responders showed an increase in FKBP5 promoter methylation over this same period (**Figure 2C**). This interaction effect was confirmed at a trend level of significance using the sum % methylation measure ($F_{2,22} = 4.276$, $p = 0.063$, trend; **Figure 2D**) and was likely due to decreased levels of FKBP5 promoter methylation among responders from post-treatment to the follow-up time-point.

In contrast to GR exon 1F promoter methylation, for which measures at pre-treatment predicted symptoms or biological measures at subsequent time-points, FKBP5 promoter methylation tended to associate cross-sectionally with biological measures at pre- and post-treatment time-points. For example, FKBP5 promoter methylation at pre-treatment was significantly correlated with plasma cortisol levels (**Figure 6A**) such that higher FKBP5 promoter methylation was correlated with lower cortisol levels at pre-treatment, a result compatible with our previous findings of lower FKBP5 gene expression in PTSD (21). Following treatment, FKBP5 promoter methylation was significantly negatively correlated with pituitary response to dexamethasone as measured by ACTH levels following the administration of low dose dexamethasone (**Figure 6B**). A similar correlation was observed at a trend level of significance with post-dexamethasone cortisol ($r = -0.509$, $n = 15$, $p = 0.053$). Since lower ACTH or cortisol levels following the low dose DST reflect a greater negative-feedback inhibition of the HPA-axis, these findings suggest that greater GR responsiveness associated with higher levels of FKBP5 promoter methylation.

Levels of FKBP5 promoter methylation at follow-up were also associated with measures of both endocrine function and symptoms at post-treatment (**Figure 7**). Thus, FKBP5 promoter methylation at follow-up was significantly correlated with both plasma cortisol and 24 h-urinary cortisol at post-treatment (**Figures 7A,B**, respectively), suggesting that FKBP5 promoter methylation may be associated with changes in HPA-axis activity, in association with changes in symptom expression, rather than reflecting upstream regulation of cortisol. The finding that post-treatment PTSD severity was correlated with FKBP5 promoter methylation at follow-up (**Figure 7C**) is consistent with this idea.

EXPRESSION OF GR EXON 1F AND FKBP5

Because the assays for gene expression were not planned at the outset of this clinical trial, biological material was only available at the follow-up time-point and not for all subjects. Treatment responders showed higher expression of the GR exon 1F and FKBP5 genes compared with non-responders (for GR exon 1F: 1.0 ± 0.1 and 0.4 ± 0.1, respectively, for FKBP5 exon 8/9: 3.1 ± 1.4 and 1.2 ± 0.4, respectively). This difference was significant only for GR exon 1F ($t_4 = 2.29$, $p = 0.019$) in this small sample.

Plasma cortisol was positively correlated with FKBP5 gene expression (for exon 7/8 transcript: $r = 0.654$, $n = 10$, $p = 0.040$) and negatively correlated with GR exon 1F expression ($r = -0.853$, $n = 6$, $p = 0.031$) at follow-up. FKBP5 gene expression also negatively correlated with the decline in cortisol in response to dexamethasone (for the exon 7/8 transcript: $r = -0.869$, $n = 10$, $p = 0.002$).

Endocrine markers assessed at pre- and post-treatment correlated with FKBP5 gene expression at follow-up. For example, ACTH levels following dexamethasone at pre-and post-treatment predicted lower GR exon 1F, and higher FKBP5 gene expression at the 7/8 transcript (at pre-treatment, $r = -0.929$, $n = 6$, $p = 0.022$ for GR exon 1F and $r = 0.712$, $n = 9$, $p = 0.031$ for the FKBP5 exon 7/8 transcript; at post-treatment $r = -0.616$, $n = 6$, ns for GR exon 1F and $r = 0.768$, $n = 10$, $p = 0.016$ for the FKBP5 exon 8/9 transcript). This suggests that those showing relatively lower pituitary GR responsiveness before and/or after treatment were most likely to demonstrate a treatment (or symptom) induced decrease in GR gene expression and/or increase in FKBP5 gene expression, a likely consequence of demethylation of the FKBP5 promoter region described above.

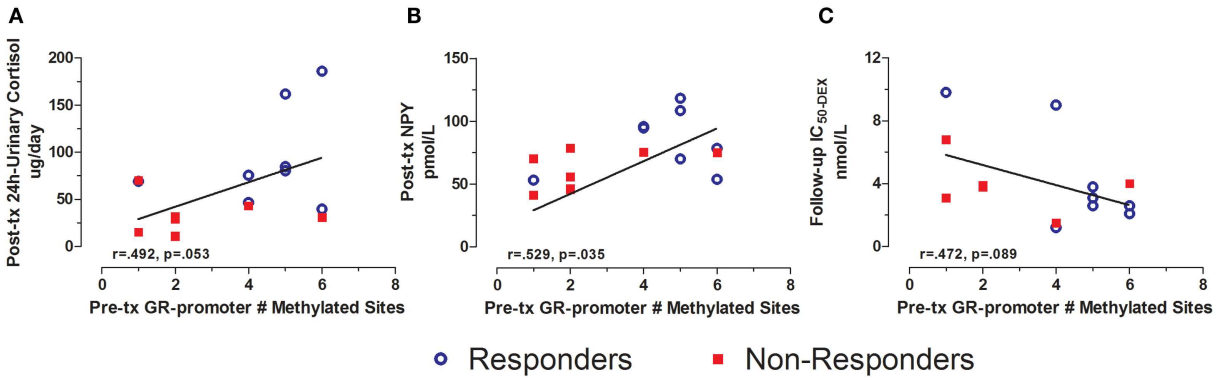

FIGURE 5 | Relationship between GR exon 1F promoter methylation at pre-treatment and urinary cortisol, NPY, and IC50-DEX at post-treatment or follow-up. Correlations of pre-treatment GR exon 1F promoter methylation (# of methylated sites) with adjusted 24 h-urinary cortisol (see below) at post-treatment **(A)**, neuropeptide-Y (NPY) at post-treatment **(B)**, and $IC_{50\text{-}DEX}$ at follow-up **(C)**. Responders ($n = 8$) to treatment are represented by blue, open circles and non-responders ($n = 8$, $n = 6$ for $IC_{50\text{-}DEX}$) by red squares. The higher number of GR exon 1F promoter methylated sites at Pre-treatment corresponded to higher adjusted 24 h-urinary cortisol and NPY at Post-treatment and lower $IC_{50\text{-}DEX}$ at follow-up (trend). About 24 h-urinary cortisol was adjusted for BMI and gender using linear regression and adding unstandardized residuals to the initial raw levels. Correlation coefficients are denoted in the different panels. Statistical significance was set at $p < 0.05$.

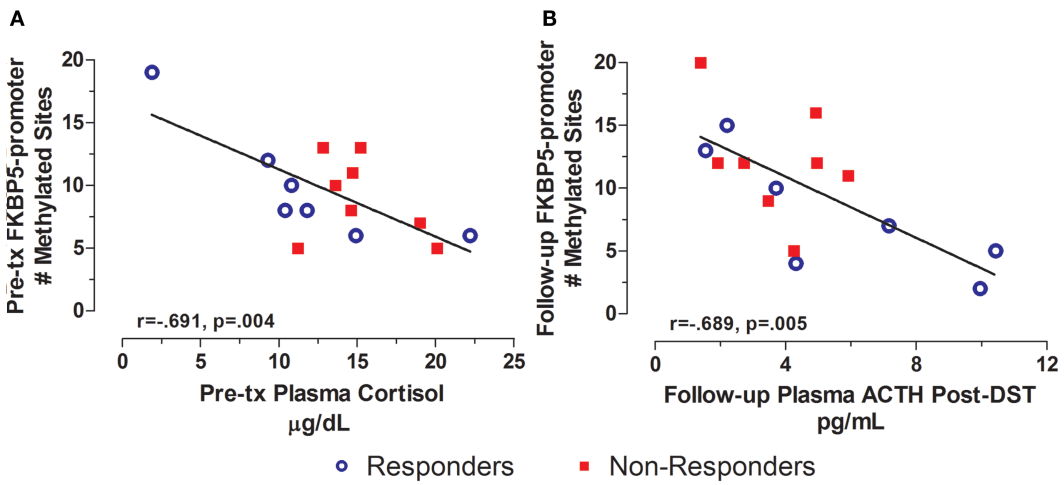

FIGURE 6 | Cross-sectional relationship between FKBP5 promoter methylation and HPA-axis endocrine markers. Correlations of pre-treatment plasma cortisol with pre-treatment FKBP5 promoter methylation (# of methylated sites) **(A)** and follow-up adjusted post low dose dexamethasone suppression test (DST) plasma ACTH with follow-up FKBP5 promoter methylation (# of methylated sites) **(B)**. Responders ($n = 7$) to treatment are represented by blue, open circles and non-responders ($n = 8$) by red squares. Higher pre-treatment plasma cortisol and Follow-up post-DEX adjusted ACTH associated with lower FKBP5 promoter methylation at pre-treatment and Follow-up, respectively. Follow-up post-DST ACTH was adjusted for dexamethasone levels and pre-DST ACTH levels using linear regression and unstandardized residuals were added to the initial raw levels. Correlation coefficients are denoted in the different panels. Statistical significance was set at $p < 0.05$.

METHYLATION AND TRAUMATIC LIFE EVENTS

There was a significant difference in total life events in responders vs. non-responders (**Table 1**). GR exon 1F promoter methylation at pre-treatment was significantly associated with time since the first reported trauma, and at post-treatment with DRRI total life events (**Figures 8A,B**, respectively). There was no relationship between FKBP5 promoter methylation (at any time-point) with either the total number of negative life events or time since initial trauma.

Interestingly, DRRI total life events predicted PTSD symptom severity assessed by CAPS at post-treatment ($r = 0.690$, $n = 16$, $p = 0.003$). The three associations did not appreciably change when controlling for participant age at the time of the GR exon 1F promoter methylation assessment.

DISCUSSION

This is the first report in the literature to investigate cytosine methylation changes in association with changes in psychiatric symptoms and neuroendocrine measures in response to psychotherapy. In this small sample of responders and non-responders to PE psychotherapy, pre-treatment GR exon 1F

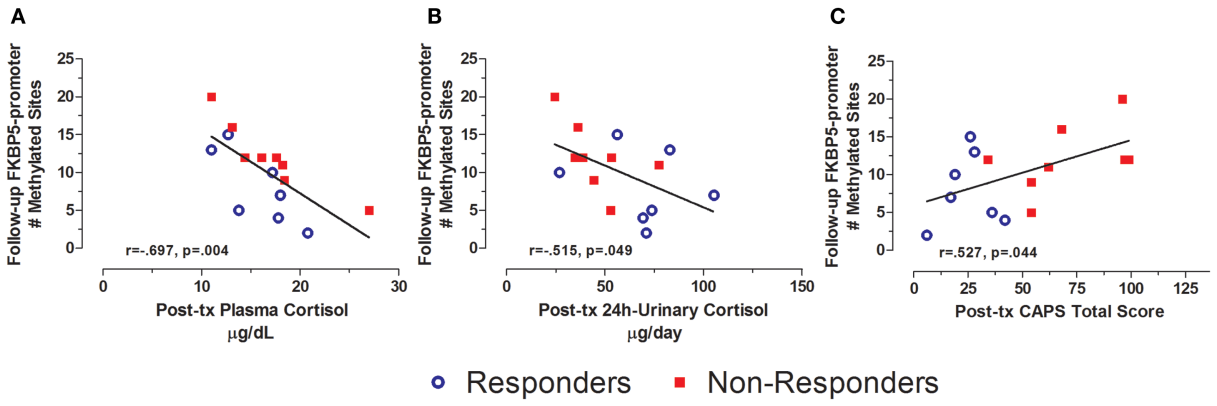

FIGURE 7 | Relationship between HPA-axis endocrine markers and PTSD severity at post-treatment and FKBP5 promoter methylation at follow-up. Correlations of post-treatment plasma cortisol **(A)**, post-treatment 24-h urinary cortisol **(B)**, and post-treatment CAPS total score **(C)** with Follow-up FKBP5 promoter methylation (# of methylated sites). Responders ($n = 7$) to treatment are represented by blue, open circles and non-responders ($n = 8$) by red squares. Both higher plasma cortisol and higher 24 h-urinary cortisol associated with lower FKBP5 promoter methylation at follow-up. Higher post-treatment CAPS total scores associated with more FKBP5 promoter methylation at follow-up. Correlation coefficients are denoted in the different panels. Statistical significance was set at $p < 0.05$.

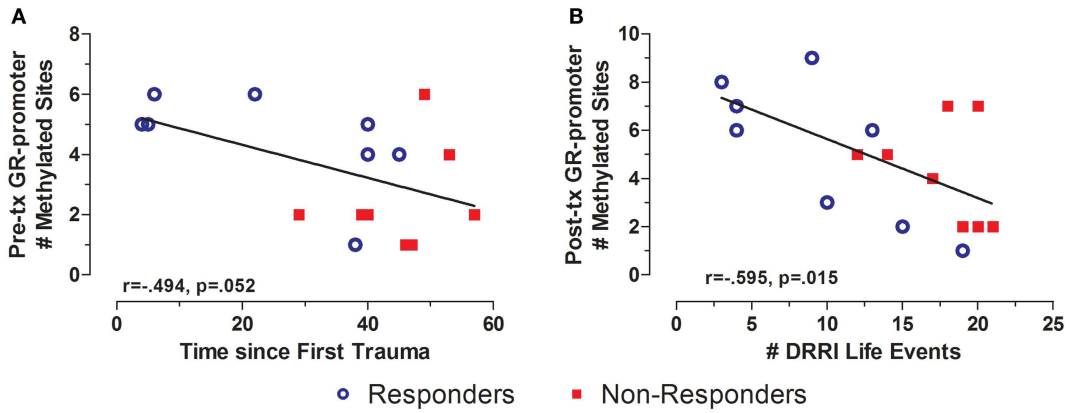

FIGURE 8 | Relationship between significant and potentially traumatic events at pre-treatment and GR exon 1F promoter methylation. Correlations of time since first trauma assessed on the Deployment Risk and Resiliency Inventory (DRRI) with the with Pre-treatment GR exon 1F promoter methylation **(A)** and number of life events as assessed on the DRRI with GR exon 1F promoter methylation at post-treatment **(B)**. Responders ($n = 8$) to treatment are represented by blue, open circles and non-responders ($n = 8$) by red squares. Longer time since first trauma and greater number of traumatic life events both associated with lower GR exon 1F promoter methylation, at pre-treatment and post-treatment, respectively. Correlation coefficients are denoted in the different panels. Statistical significance was set at $p < 0.05$.

promoter methylation predicted treatment outcome, but was not significantly altered in either group at post-treatment or follow-up. In contrast, pre-treatment cytosine methylation of the FKBP5 promoter did not predict treatment response, but decreased in association with recovery in veterans who no longer met diagnostic criteria for PTSD after psychotherapy. These findings distinguish two seemingly stable epigenetic markers that may associate, respectively, with prognosis (GR gene methylation) and symptom severity (FKBP5 gene methylation).

The focus in this study on the GR gene was based on observations that implicate enhanced GR sensitivity in PTSD (36). The focus on FKBP5, a co-chaperone of the GR cellular complex, is based on studies showing that FKBP5 inhibits the nuclear translocation of ligand-bound GR, thereby directly affecting functional GR sensitivity (12). FKBP5 gene expression is up-regulated by glucocorticoids through consensus glucocorticoid response elements (GREs) and by glucocorticoid-induced demethylation of the gene (12, 17, 52, 53). The underlying epigenetic mechanisms involved in the interaction of these two genes are not fully known, and remain of great interest. The findings of this study demonstrate distinct correlates with respect to PTSD for these two glucocorticoid related genes.

The lack of change over time in GR gene methylation is consistent with the idea that imprinting by early environmental

experiences may result in enduring epigenetic changes in expression of this gene (1, 54). In animals, changes in GR gene methylation related to variations in maternal care are enduring, predicting GR responsiveness under a variety of experimental challenges in adulthood (5, 7, 55). Similarly, child maltreatment associates with hypermethylation of the GR exon 1F promoter in both post-mortem hippocampus (9) and leukocytes and an attenuated cortisol response to the Dex/CRH test in healthy adults (40). Our findings are consistent with the idea that environmental influences on the methylation of the GR exon 1F promoter are stable into adulthood and associated with clinical outcomes. Thus, methylation of this GR promoter was relatively stable across a 6-month period during which three independent measures were obtained under circumstances of changing symptom severity. This conclusion is buttressed by the strong inverse correlation between total number of negative life events as measured by the DRRI and GR gene 1F promoter methylation, as well as by the negative relationship between GR gene methylation and duration since initial trauma exposure. To our knowledge this finding is the first systematic documentation in humans of the stability of an epigenetic mark associated with childhood experience. In contrast, studies with rodents have been limited in most studies to a single assessment, typically in selected brain regions.

Variations in maternal care regulate hippocampal GR promoter methylation that, in turn, determines hippocampal GR expression, the efficiency of glucocorticoid negative-feedback regulation of hypothalamic CRF expression, and the magnitude of HPA-axis responses to stress (5, 56, 57). GR promoter methylation is thus an upstream regulator of GR gene expression and HPA-axis responsivity. In this study, GR promoter methylation at pre-treatment predicted HPA-axis activity following psychotherapy, but was not correlated with baseline cortisol measures at pre-treatment. Likewise in a recent cross-sectional study (40), GR promoter methylation in human lymphocytes did correlate with the cortisol response to the DEX/CRH challenge in a sample of healthy adults, many of whom had reported child abuse, but apparently not with basal measures of cortisol. It is possible, that cross-sectional correlations would have been observed herein following a corresponding level of HPA-axis manipulation as implicated by the DEX/CRF challenge. Nonetheless, the associations between GR exon IF promoter methylation and numerous functional glucocorticoid measures further increase confidence in the validity of the former to inform downstream processes related to functional neuroendocrine outcomes, but these associations may not necessarily be present when examined cross-sectionally.

Unlike GR promoter methylation, FKBP5 promoter methylation did not predict treatment response, but was correlated with measures of cortisol and glucocorticoid sensitivity. These findings are consistent with the role of FKBP5 as a moderator of intracellular GR signaling. These results are also consistent with our previous findings of an association between FKBP5 gene expression and plasma cortisol levels in WTC trauma survivors (21). The FKBP5 site in the current study differs from that examined by Klengel et al. (17), which associates with childhood adversity. This group examined regions (intronic regions and distal promoter region) of the FKBP5 gene that contain GREs. Methylation status in the intronic regions, especially of intron 7, mediated the effects of early life adversity on adult stress sensitivity since an association of child abuse with FKBP5 methylation at intron 7 has been reported, depending on FKBP5 genotype (17). Interestingly, methylation at these respective intronic regions in the rat, which also contain GREs, were decreased after a month of corticosterone administration resulting in an increase in FKBP5 gene expression strengthening the link between glucocorticoid levels and FKBP5 gene expression through epigenetic mechanisms that can also operate later in life (52, 53). Our choice of examining the proximal promoter region was prompted by the notion that methylation of this region would influence FKBP5 gene expression. Consistent with the findings of Klengel et al. (17), we found that increased levels of cortisol associated with decreased levels of FKBP5 promoter methylation.

These findings permit a distinction between biological markers associated with prognosis and treatment outcome. Thus, GR promoter methylation at pre-treatment was associated with treatment response, while dynamic variation in FKBP5 promoter methylation associated with treatment outcome. A model for understanding the unique relationships observed in GR and FKBP5 methylation and their potential interactions in PTSD is presented in **Figure 9**. Early experience may influence both GR and FKBP5 gene methylation. In PTSD, GR sensitivity is increased, likely resulting from reduced GR promoter methylation, which would ultimately result in lowered cortisol levels and, therefore, low glucocorticoid signaling. The low cortisol levels would serve to further decrease FKBP5 gene expression through an intracellular loop mediated by GREs in the FKBP5 gene. Decreased FKBP5 gene expression could serve to sustain an increased GR sensitivity. A decline in FKBP5 promoter methylation, such as occurred in treatment responders, might allow for an increase in FKBP5 gene expression, which would, in turn, ultimately decrease GR sensitivity. Thus, we found that treatment responders showed decreased FKBP5 promoter methylation, suggestive of increased FKBP5 gene expression, and measures of HPA-axis activity (i.e., plasma and urinary cortisol levels) reflecting decreased GR sensitivity. Likewise, higher levels of GR promoter methylation, suggestive of lower GR expression, were also associated with a positive response to treatment. Our previous studies suggest that increased GR sensitivity is a hallmark of PTSD (36). The mechanisms by which such dynamic changes in GR sensitivity associate with changes in PTSD symptoms remains to be fully elucidated; however the current findings suggest that the molecular mechanisms that regulate glucocorticoid signaling associate with treatment outcome.

In sum, this is the first demonstration of an epigenetic alteration in association with treatment response. This study represents an important initial step in establishing relevant molecular markers for PTSD therapies. In particular, the longitudinal approach in which symptoms vary over time is essential to distinguishing PTSD predictors from symptom correlates, and permits a more rational evaluation of potential treatment targets. The preliminary observations presented here require replication. Future prospective studies could detect the level of functional significance of small differences in methylation at baseline (as was the case for the GR-1F promoter) or small changes in methylation after an environmental challenge (as was noted for FKBP5 promoter

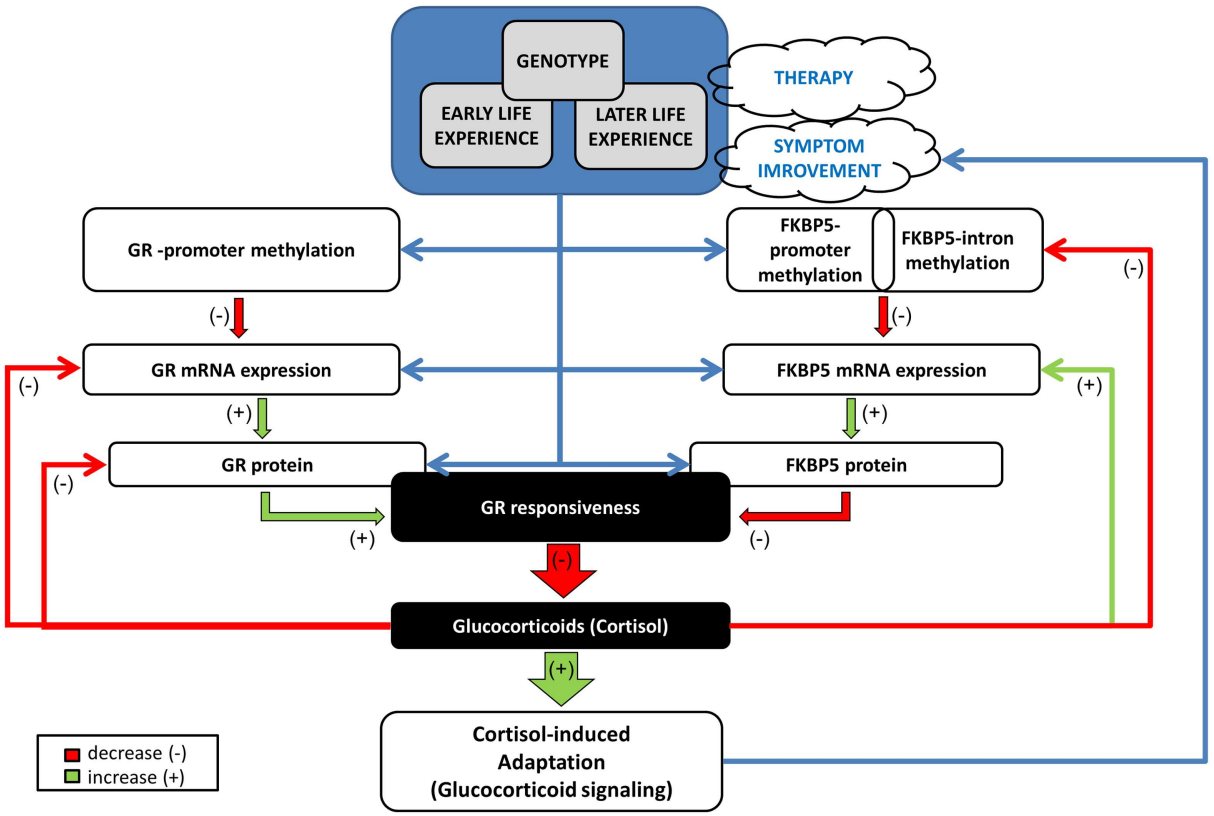

FIGURE 9 | Mechanistic model of the relationships/interactions between GR and FKBP5 methylation in PTSD. Early life experience may impact both GR and FKBP5 gene methylation potentially in interaction with genotype. GR responsiveness is increased in PTSD, likely resulting from reduced GR methylation, with consequent increased GR expression, ultimately resulting in lowered cortisol levels. Low cortisol levels would serve to decrease FKBP5 gene expression through an intracellular loop mediated by GREs in the FKBP5 gene. Later life experience ± therapy, can also impact methylation of both genes, but most likely in distinct manners. A glucocorticoid-induced demethylation of FKBP5 will allow the subsequent increase in FKBP5 mRNA and protein expression, which would, in turn, ultimately decrease GR responsiveness, permitting the normalization of cortisol and of glucocorticoid signaling. This would have a beneficial effect on PTSD symptoms by impacting glucocorticoid responsive DNA sites that reduce sympathetic arousal or stimulate adaptation and recovery/resiliency (e.g., neuropeptide-Y). Green arrow denotes a positive influence (increase, "+") and red arrow a negative influence (decrease, "−"). Blue arrow depicts a relationship. GR: glucocorticoid receptor encoded by the *NR3C1* gene, FKBP5: FK506 binding protein 5 encoded by the *FKBP51* gene.

methylation). However, the results support recent research indicating that some glucocorticoid related genes may be subject to environmental regulation throughout life (58). Moreover, the data suggest that psychotherapy resulting in substantial symptom change constitutes a form of "environmental regulation" that may alter epigenetic state. Finally, the results demonstrate that different genes may be associated with prognosis and symptom state, respectively.

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Epigenetic risk factors in PTSD and depression

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Epidemiological and clinical studies have shown that children exposed to adverse experiences are at increased risk for the development of depression, anxiety disorders, and posttraumatic stress disorder (PTSD). A history of child abuse and maltreatment increases the likelihood of being subsequently exposed to traumatic events or of developing PTSD as an adult. The brain is highly plastic during early life and encodes acquired information into lasting memories that normally subserve adaptation. Translational studies in rodents showed that enduring sensitization of neuronal and neuroendocrine circuits in response to early life adversity are likely risk factors of life time vulnerability to stress. Hereby, the hypothalamic-pituitary-adrenal (HPA) axis integrates cognitive, behavioral, and emotional responses to early-life stress and can be epigenetically programed during sensitive windows of development. Epigenetic mechanisms, comprising reciprocal regulation of chromatin structure and DNA methylation, are important to establish and maintain sustained, yet potentially reversible, changes in gene transcription. The relevance of these findings for the development of PTSD requires further studies in humans where experience-dependent epigenetic programing can additionally depend on genetic variation in the underlying substrates which may protect from or advance disease development. Overall, identification of early-life stress-associated epigenetic risk markers informing on previous stress history can help to advance early diagnosis, personalized prevention, and timely therapeutic interventions, thus reducing long-term social and health costs.

Keywords: PTDS, depression, early-life stress, HPA axis, epigenetic programing, epigenetic variation

INTRODUCTION

The overall burden of mental disorders – individual, societal, and economic – has been increasing in recent decades (1) and is greater than 10 years ago despite the availability of reasonably effective pharmacological and psychological interventions (2). Among mental disease posttraumatic stress disorder (PTSD) is a debilitating stress-related disease with prevalence rates amounting to 8%, and considerable higher rates among those living in high-violence areas and combat veterans (3–5). Originally thought of as a normative response to trauma, epidemiological studies showed that exposed subjects differed with respect to their later on risk for developing PTSD (6). This finding led to intense investigations to identify genetic and environmental factors associated with the onset, course, and treatment response of this disease. Overall, epidemiological and clinical studies have provided compelling evidence for a strong association between various forms of early life adversity, depression, and PTSD (**Figure 1**).

For this reason, we will refer the reader to the role of early life adversity for the development of depression whenever corresponding findings in PTSD are not available. This applies in particular to the role of experience-dependent epigenetic programing which is increasingly recognized as a mechanism in depression (7, 8). Moreover, given the central role of the hypothalamic-pituitary-adrenal (HPA) stress axis in response to early life adversity we will focus on its deregulation in both depression and PTSD. While PTSD and depression represent distinct entities, such a comparative approach can provide a conceptual framework for future studies in PTSD.

According to the National Comorbidity Survey Replication (9) early life adversity comprises interpersonal loss (parental death, parental divorce, and other separation from parents or caregivers), parental maladjustment (mental illness, substance abuse, criminality, and violence), maltreatment (physical abuse, sexual abuse, and neglect), life-threatening childhood physical illness in the respondent, and extreme childhood family economic adversity.

A landmark survey conducted at the Center for Disease Control showed a strong dose-response relationship between childhood adversities and mental health problems in adulthood (10). Patients suffering from major depression showed a fourfold increased risk for depression following multiple adverse exposures (11), a dose-response relationship between the severity of experienced childhood adversities and lifetime recurrent depression (12) and a twofold to fivefold increase in attempted suicide in up-growing children, adolescents, or later adults (13). These data corroborate with other representative surveys such as the National Comorbidity Survey (14), the Ontario Health Survey (15, 16), and the New Zealand Community Survey (17).

Similarly, individuals who were exposed to early life adversity are also more likely to develop PTSD (18, 19), to face re-exposure to trauma in adulthood, and to suffer from PTSD following trauma in adulthood (20–22) (**Figure 1**).

Hence, early life adversity is a powerful risk factor for mental diseases such as depression and PTSD and can predict a prolonged course and poorer response to treatment. The high incidence of child maltreatment represents an epidemic health problem and the

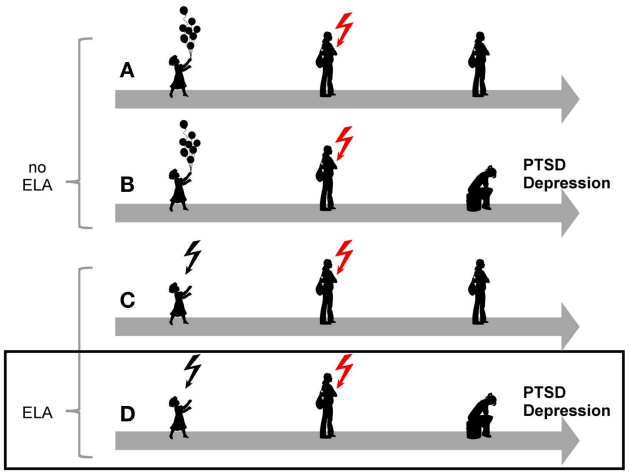

FIGURE 1 | Model for the role of early-life adversity for PTSD and depression. In the absence of a history of early life adversity (ELA), adults can be resilient to disease upon exposure to severe trauma and stress **(A)** while others will develop PTSD or depression **(B)**. Similarly, not all children exposed to early life adversity will develop disease upon anew exposure to trauma and stress **(C)**. On the other hand, exposure to early life adversity in childhood can give rise to a vulnerable phenotype predisposing to disease upon anew exposure to trauma and stress **(D)**.

long-term consequences of such trauma place a heavy burden on the healthcare system and society (23). According to the National Center of Child Abuse and Neglect approximately 3.4 million referrals, comprising alleged maltreatment of approximately 6.2 million children, were received across the US in 2011 (24). One-fifth of these children were found to be victims with disposition of substantiated (18.5%), indicated (1.0%), and alternative response victim (0.5%). All in all 676,569 victims of child abuse and neglect give rise to a unique victim rate of 9.1 victims per 1,000 children in the US population:

- more than 75% (78.5%) suffered neglect
- more than 15% (17.6%) suffered physical abuse
- less than 10% (9.1%) suffered sexual abuse
- more than four children (1,570 fatalities) died daily

These numbers recapitulated by and large prior findings (10, 25–28). Despite this compelling evidence for a link between early neglect, abuse, and later psychopathology (9, 29) knowledge about the molecular mechanisms underlying the long-term mental health consequences remains poor (30).

THE VULNERABLE BRAIN

Early brain development is a time of great opportunities and great vulnerabilities. The architecture of the developing brain is constructed through an ongoing process that begins before birth and extends into adulthood. Brain development and architecture are built from the bottom up with simple structures providing the scaffold for the formation of more advanced structures over time. Research in the field of neuroscience has provided compelling evidence for the high plasticity of the developing brain as a function of experience which allows encoding of acquired information into lasting memories that normally subserve adaptation (31, 32). Sensitive periods refer to time windows during which developmental cues induce lasting programmable and organizational effects on neuronal substrates. Early adverse experiences in rodents and higher primates such as prenatal maternal stress, maternal separation, variable foraging demand, or naturally occurring low maternal care can lead to structural and functional changes in a connected network of brain regions implicated in neuroendocrine control, autonomic regulation, and vigilance (33–36). These neural changes converge on lifelong increased physiological and behavioral responses to subsequent stress (37). Ultimately, the enduring effects of early life adversity on the brain and its regulatory outflow systems, comprising the autonomic, endocrine, and immune systems, may lead to the development of a vulnerable phenotype with increased sensitivity to stress and risk for a range of somatic (38–40) and behavioral disorders (i.e., depression and PTSD) (9).

THE CENTRAL ROLE OF THE HPA AXIS

Early life adversity can cause lasting structural and regulatory adaptations in the neuroendocrine system predisposing to or protecting from stress-related diseases later in life (41–43). Initial studies on PTSD focused on the HPA axis due to its central position in the neuroendocrine stress response (**Figure 2**). The two neuropeptides corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are secreted by the parvocellular neurons of the hypothalamic paraventricular nucleus (PVNh) and coordinate the behavioral and metabolic responses to stress. Binding to G-protein coupled receptors at the anterior pituitary gland induces co-operatively pro-opiomelanocortin (POMC) which is processed to adrenocorticotrophin (ACTH), opioid, and melanocortin peptides among others. Subsequently, ACTH stimulates the adrenal cortex to secrete cortisol (in humans) and corticosterone (in humans, rats, and mice). Corticosteroids activate ligand-gated glucocorticoid (GR) and mineralocorticoid (MR) receptors which are coexpressed in neurons of limbic structures. High levels of GR are additionally detected in the PVNh and the anterior pituitary. The MR is thought to serve in the assessment and onset of the stress response whereas GR, requiring higher amounts of glucocorticoids for activation, terminates the stress reaction (44).

In contrast to the PVNh, CRH neurons in the central nucleus of the amygdala project to the locus coeruleus and enhance the firing rate of its neurons resulting in increased noradrenaline release in the vast terminal fields of the ascending noradrenergic system (45). One of the principal noradrenergic targets are the CRH neurons of the PVNh, which respond to increased activity in the nucleus tractus solitarius, the dorsal medullary nucleus, and the locus coeruleus (46). Experimental lesions of these regions result in a significant decrease in stress-induced PVNh levels of noradrenaline, reduced plasma ACTH responses, and behavioral responses to stress (47, 48). In sum, CRH neurons in the PVNh and the central nucleus of the amygdala jointly mediate behavioral and endocrine responses to stress.

Although commonly adaptive, sustained activation of these stress circuits results in impairments. Elevated central CRH activity is associated with symptoms of anxiety and depression. This

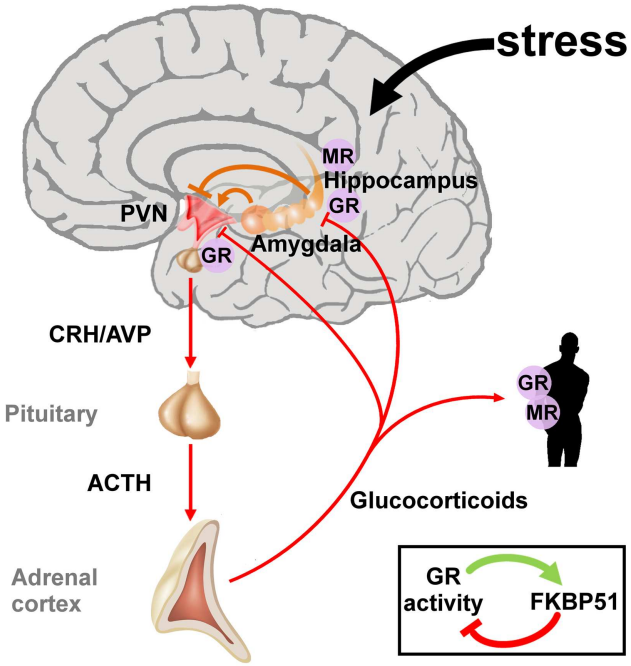

FIGURE 2 | The hypothalamic-pituitary-adrenal axis integrates and mediates the stress response to early life and later on adversity. The perception of real and/or presumed physical and social threats causes activation of the hypothalamic-pituitary-adrenal axis. Anxious states arise from activation of the amygdala and magnify the stress response via neuronal projections to the paraventricular nucleus (PVN). The hippocampus plays an important role in the assessment of stressors and as a site of glucocorticoid receptor (GR) mediated negative feedback regulation. Release of the hypothalamic neuropeptides corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) promotes the synthesis and secretion of adrenocorticotrophin (ACTH), a posttranslational cleavage product of anterior pituitary pro-opiomelanocortin mRNA (POMC). ACTH in turn stimulates the release of glucocorticoids from the adrenal glands. These hormones circulate throughout the whole body and the brain and bind to intracellular nuclear steroid receptors. Hippocampal mineralocorticoid (MR) receptors act to the onset of the stress response, while GR at the hippocampus, PVN, and anterior pituitary terminates the stress response. The GR further transactivates FKBP51 encoding a chaperon protein which curtails GR activity through a fast intracellular negative feedback loop.

is most likely due to the CRH-induced stimulation of serotonergic and noradrenergic systems (49). Glucocorticoids and catecholamines enhance learning for information related to the stressor (50) whereas glucocorticoids impair attention and learning in relation to events not directly associated with the stressor (37). Additionally, glucocorticoids facilitate the behavioral effects of central Crh partly due to the upregulation of Crh in the central nucleus of the amygdala (51, 52).

LONG-TERM EFFECTS OF EARLY-LIFE ADVERSITY

In rodents and higher primates early-life stress lastingly influences the development of the central CRH systems underlying the expression of behavioral, emotional, autonomic, and endocrine responses to stress later in life (53, 54). Major depression frequently shows signs of a disinhibited HPA axis due to an increased parvocellular CRH expression which drives production and secretion of pituitary ACTH and subsequently cortisol (**Table 1**). CRH overexpression reflects an impaired negative GR feedback regulation as evidenced by a reduced inhibition of cortisol secretion after application of the synthetic glucocorticoid dexamethasone (55) (**Table 1**).

Notably, depressed patients with a history of early child adversity were shown to differ in their neuroendocrine regulation. Although they shared signs of a hyperactive HPA axis, plasma cortisol is unaltered or reduced in part of these patients (**Table 1**). Moreover, their response to psychotherapy alone appeared superior to antidepressant therapy when compared to patients without a history of early child adversity (56).

Adult PTSD patients show a distinct neuroendocrine profile characterized by centrally elevated CRH, low plasma cortisol, and enhanced suppression of plasma cortisol and ACTH following the dexamethasone suppression test (**Table 2**). An increased responsiveness of both peripheral and central GR has been suggested as a plausible cause for hypocortisolemia (**Table 2**).

While hypocortisolemia in depressed patients is confined to those with a history of early life adversity (**Table 1**, right), PTSD patients are affected in either case (**Table 2**, left). This raises the question of whether hypocortisolemia precedes trauma exposure or whether it is a long-term consequence thereof promoting the manifestation of either depression or PTSD.

In this regard, longitudinal studies have shed some light on the development of HPA-axis deregulation in traumatized children. From an early age on, maltreatment, neglect, physical, and sexual abuse manifest with low levels of cortisol which persist into adulthood (**Table 2**, right). Furthermore, abused children display flattened diurnal cortisol levels (57) and increased cortisol suppression after administration of dexamethasone (58). These results corroborate the concept of an enhanced GR response and negative feedback function. However, sexually abused girls initially showed higher levels of cortisol after trauma exposure. Hypercortisolemia decreased, however, during growing up and cortisol remained low in young adults when compared to their unabused peers (59). A similar cortisol pattern has been detected in pediatric patients who experienced motor vehicle accidents and developed PTSD at 6 months. Initially, they had high levels of cortisol which passed over into hypocortisolemia at 6 months (60). Taken together, these findings suggest that trauma exposure associates with an acute stress response followed by hypocortisolemia and GR hyperresponsiveness in vulnerable subjects. This raises the important question of whether hypocortisolism precedes clinical manifestation of PTSD and thus represents a potential marker for later on risk for illness (**Table 2**). In this respect, longitudinal studies identified low levels of cortisol as a risk factor to develop PTSD after trauma exposure (61–63).

In conclusion, neuroendocrine findings in PTSD and individuals exposed to early life adversity indicate that hypocortisolemia *per se* does not result in PTSD but seems to increase the risk to develop disease following additional trauma exposure. The cellular and molecular mechanisms causing hypocortisolemia due to enduring deregulation of the HPA axis and how they interact with later on trauma to manifest as PTSD are presently largely unknown.

Table 1 | Neuroendocrine status in depressed patients without and with a history of early life adversity.

	MD	Reference	MD + ELA	Reference
CRH	↑	Nemeroff et al. (139)	↑	Carpenter et al. (140)
	↑	Raadsheer et al. (141)		
	↑	Wang et al. (142)		
Cortisol	↑	Sachar et al. (143)	↓	Shea et al. (144)
	↑	Holsboer et al. (145)	↓	Heim et al. (146)
	↑	Holsboer et al. (147)	↑	Gerra et al. (148)
	↑	Wingenfeld et al. (149)	=	Heim et al. (150)
	↑	Carvalho Fernando et al. (151)	↑	Power et al. (152)
	↑	Hinkelmann et al. (153)		
	↑	Messerli-Bürgy et al. (154)		
	↑	Yilmaz et al. (155)		
DST	Cortisol ↑	Carroll et al. (156)	ACTH↓ cortisol ↓	Newport et al. (157)
	ACTH ↑ cortisol ↑	Modell et al. (158)		
	Cortisol ↑	Carvalho Fernando et al. (151)		
CST	ACTH ↑	Holsboer et al. (147)	ACTH ↓ cortisol ↓	Heim et al. (146)
	ACTH ↓	Heim et al. (146)	Cortisol ↑	Heim et al. (150)

MD, major depression; ELA, early life adversity; DST, dexamethasone suppression test; CST, corticotrophin-releasing hormone stimulation test; ACTH, adrenocorticotrophin hormone.

In the following parts, we will explore the possibility of epigenetic mechanisms to encode traumatic experiences. We will further argue that insight from experience-dependent epigenetic programing by early-life adversity in depression can serve as guidance to advance translational studies in PTSD.

A GUIDE TO DNA METHYLATION

The epigenetic landscape has unfolded at multiple layers involving DNA methylation, histone modifications, non-coding RNA, and nucleosome positioning; along with DNA sequence. For the purpose of this review we will consider only the former two and refer the interested reader to recent reviews (64–66).

As a stable, though potentially reversible, repressive mark, DNA methylation is considered to represent an important player in epigenetic control of transcription (67). DNA methylation is carried out by a family of DNA methyltransferases (DNMTs) comprising DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT1 primarily methylates hemimethylated DNA *in vitro* and is recruited to replication foci during S phase, while DNMT3A and DNMT3B preferentially recognize unmethylated CpG dinucleotides and regulate *de novo* methylation during development.

Specific histone modifications and DNA methylation reciprocally influence each other in deposition (**Figure 3**). Mechanistically, components of the histone methylation system associate physically with one or more DNMTs. For example, trimethylation of H3K9, H3K27, and H4K20 emerged as a prerequisite for subsequent DNA methylation. The methyltransferases SUV39H1/2 and EZH2 catalyzing H3K9 and H3K27 methylation, respectively, bind directly to DNMT1, DNMT3A, or DNMT3B and facilitate their recruitment to target loci (68, 69). Similarly, the recruitment of HP1 (heterochromatin protein 1) to constitutive heterochromatin results from SUV39H1/2-mediated trimethylation of H3K9 while euchromatin binding depends on dimethylation catalyzed by the histone methyltransferase G9A (70). Once bound, HP1 can directly interact with DNMT3A to guide DNA methylation (71). Conversely, histone methylation can also interfere with DNA methylation. DNMT3L, lacking catalytic activity, specifically recognizes the extreme amino terminus of the core histone H3 in a methylation sensitive manner. Methylation of H3K4 but not of other residues blocks interaction with DNMT3L and the subsequent recruitment of DNMT3A2 (72).

Taken together, histone methylation can both facilitate and impair the recruitment of DNMTs and intermediary factors in a context dependent manner to impose changes in DNA methylation and the long-term control of gene activity (**Figure 3**).

The globally methylated, CpG poor genomic landscape is punctuated by CpG islands (CGIs). Approximately 70% of all annotated gene promoters are associated with a CGI that are typically free of DNA methylation (73). In mammals tissue- and cell type-specific DNA methylation is detected in a small percentage of 5′ CGI promoters whereas most DNA methylation occurs in intra- or inter-genic regions which unexpectedly correlates with increased rather than decreased transcription (74–76) or alternatively with elective promoter usage (77).

Active DNA demethylation in mammals remains presently subject of intense investigation as cytosine deamination, oxidation, and base excision repair enzymes have been suggested in a variety of combinations (78).

A second, more recently discovered mechanism implicates the actions of ten eleven translocation (TET) proteins which convert 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) by an oxygen-, and α-ketoglutarate-dependent mechanism, then to 5-formylcytosine and hereafter to 5-carboxycytosine (79). This modified residue is excised by glycosylases to be replaced by

Table 2 | Neuroendocrine status in PTSD patients without and with a history of early life adversity and in healthy controls exposed to early life adversity.

	PTSD	Reference	ELA	Reference
CRH	↑	Bremner et al. (159)	↑	Lee et al. (160)
	↑	Baker et al. (161)		
	↑	de Kloet et al. (162)		
Cortisol	↑→ ↓ *Ch*	Pervanidou et al. (60)	↓ *Ch*	Carlson and Earls (163)
	↓ *ELA*	Bremner et al. (164)	↓ *Ch*	King et al. (165)
	↓ *ELA*	Brand et al. (166)	↓ *Ch*	Kliewer (167)
	↓	Yehuda et al. (168)	↓ *Ch*	Bevans et al. (169)
	↓	Boscarino (170)	↓ *Ch*	Badanes et al. (171)
	↓	Yehuda et al. (172)	↓ *Ch*	Hauser et al. (173)
	↓	Thaller et al. (174)	≈*Ch*	Bruce et al. (57)
	↓	Rohleder et al. (175)	↑ *Ch*→ ↓	Trickett et al. (59)
	↓	Bierer et al. (176)	↓	Resnick et al. (61)
	↓	Brand et al. (177)	↓	Brewer-Smyth and Burgess (178)
	↓	Wingenfeld et al. (149)	↓	Flory et al. (179)
	↓ *RF*	Resnick et al. (61)	↓	Power et al. (152)
	↓ *RF*	McFarlane et al. (62)	≈*Ad*	Weissbecker et al. (180)
	↓ *RF*	Delahanty et al. (63)	≈*Ad*	van der Vegt et al. (181)
GR-r	↑	Yehuda et al. (182)	↑	Heim et al. (146)
	↑	Yehuda et al. (183)		
	↑	Yehuda et al. (184)		
DST	Cortisol ↓	Yehuda et al. (183)	Cortisol ↓ *Ch*	Goenjian et al. (58)
	ACTH↓ *ELA*	Duval et al. (185)	Cortisol ↓	Stein et al. (186)
	ACTH↓ cortisol↓	Yehuda et al. (187)	Cortisol ↓	Heim et al. (188)
	ACTH↓	Ströhle et al. (189)		
CST	ACTH↓ cortisol↓	Ströhle et al. (189)	Cortisol ↑	Heim et al. (188)
			Cortisol ↓	Carpenter et al. (190)
			ACTH ↑	Heim et al. (146)

PTSD, posttraumatic stress disorder; ELA, early life adversity; GR-r, glucocorticoid receptor reactivity; DST, dexamethasone suppression test; CST, corticotrophin-releasing hormone stimulation test; ACTH, adrenocorticotrophin hormone. Indices are; RF, risk factor for PTSD; Ch, children; ≈Ch or ≈Ad, flattened diurnal cortisol in children or adults.

cytosine via the base repair system (80). Interestingly, hydroxymethylation can be selectively recognized by DNA-binding proteins raising the prospect that it serves a biological function on its own (81). For example, Mbd3 preferentially binds to 5-hmC relative to 5-methylcytosine to confer gene repression (82).

In sum, DNA methylation occurs at different gene regulatory regions controlling transcription, alternative promoter usage, and splicing. Methylation of CpG residues is a stable though potentially reversible mark catalyzed by enzymatic conversion.

AN EPIGENETIC ARCHIVE

The "DNA ticketing theory of memory" was proposed some 40 years ago by Griffith and Mahler to account for the extraordinary persistence of memory (83). Given that "nerve cells do not normally divide in adult life and their DNA is generally considered to be metabolically stable" they hypothesized that "the physical basis of memory could lie in the enzymatic modification of the DNA of nerve cells" whereby "the modification consists of methylation (or demethylation)." This concept has been reviewed by Holliday who proposed that "the initial signal which is to be memorized, switches the DNA from a modified to an unmodified state, or vice versa;" thereby, "demethylation is a more likely possibility since from existing evidence this may activate a gene" (84). This hypothesis has been largely ignored because DNA methylation was originally thought of as part of a stable, epigenetic cellular memory system that controls gene silencing via chromatin structure. Hence, postmitotic cells were considered to be refractory to any changes in DNA methylation. Recent reports suggest, however, that DNA methylation is also involved in controlling the dynamics and plasticity of gene regulation, particularly during differentiation (85). Moreover, DNA methylation in postmitotic neurons can respond to social experiences and encode lasting changes in gene expression (86). In this regard, experience-dependent DNA memories can be described as the sequential process of a social and/or physical event registered by sensory and cognitive brain areas. The subsequent activation of intracellular signaling pathways, coupled to the epigenetic machinery, and ultimately its recruitment to specific gene loci, enables to erase, establish, and maintain epigenetic

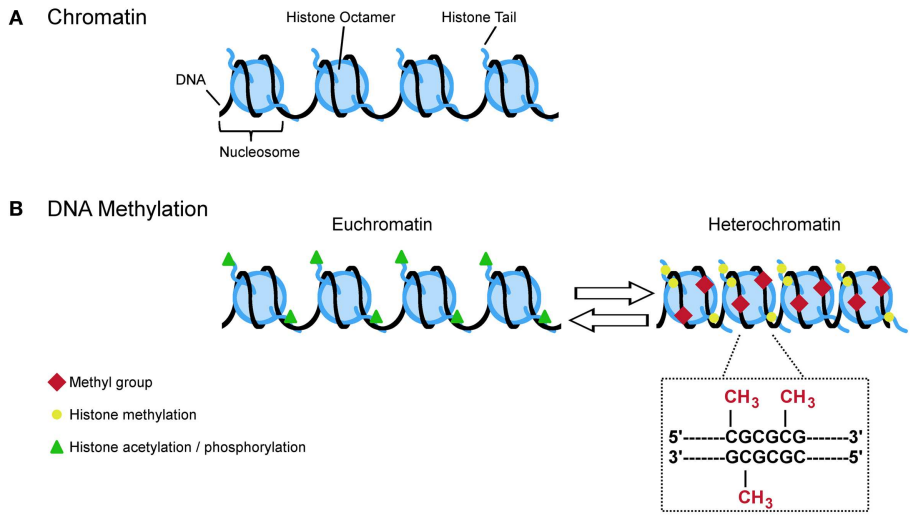

FIGURE 3 | A (simplified) guide to DNA methylation. **(A)** Approximate 147 bp of DNA are wrapped around the nucleosome which is made up by the core histones H2A, H2B, H3, and H4. The free ends of the histone tails serve as substrate for posttranslational modifications (e.g., acetylation, phosphorylation, and methylation) that influence the configuration of chromatin. Opened states (euchromatin) allow transcription while closed states (heterochromatin) restrict the transcriptional machinery. **(B)** Euchromatin is characterized by acetylation and phosphorylation (green triangles) whereas methylation (yellow circles) is more often found at heterochromatin. Specific histone modifications and DNA methylation reciprocally influence each other in deposition. For example, histone methylation at H3K9, H3K27, and H4K20 promotes DNA methylation at CpG dinucleotides. This covalent modification refers to the transfer of methyl group to cytosine resides at gene regulatory regions. Hypermethylation typically links to lasting transcriptional repression while hypomethylation favors gene expression.

marks. Admittedly, not all examples of early-life stress-dependent changes in DNA methylation can satisfy these criteria (7).

For the purpose of this review, we will focus on those genes of the HPA axis known to be lastingly deregulated in both depression and PTSD and ask whether epigenetic programing contributes to these processes. Experience-dependent programing of the HPA axis has been intensively studied in rodents and examples given below serve to illustrate the overarching concept without attempting to provide a comprehensive survey of this field. In considering epigenetic programing of key regulators of the HPA axis, a developmental perspective (i.e., embryonic, prenatal, postnatal, and adult stages) is adopted whenever possible to illustrate that the brain is susceptible to epigenetic programing across the entire life span.

EXPERIENCE-DEPENDENT PROGRAMING OF CRH

Parvocellular hypothalamic CRH-expressing neurons participate both in the peripheral and central stress systems by governing secretion of ACTH from the pituitary contributing to the peripheral, neuroendocrine stress response, and by modulating stress-related behavior including anxiety as well as learning and memory. The CRH peptide is additionally detected in neocortex and in limbic regions including the central nucleus of the amygdala, the dorsal and ventral part of the bed nucleus of the stria terminalis, and the hippocampus. Further sites comprise the locus coeruleus, dorsal and median raphe, periaqueductal gray, nucleus of the solitary tract, and cerebellar complex. For the purpose of this review, we will focus in the following section largely on stress-dependent programing of Crh in the PVNh.

Early-life experience can evoke lasting changes in Crh expression levels in the PVNh as evidenced by several paradigms comprising onetime or repeated separation from the mother for up to 24 h, manipulation of maternal behavior by "handling" and limiting nesting materials for the dam during the first weeks of life. In this respect, maternal separation for 24 h has been documented to either not influence (87) or reduce basal C*rh* gene expression in rat (88) and mice PVNh (89). Of note, prolonged maternal separation for 8 h leads to desensitization of the HPA axis with reduced corticosterone after replication of prolonged maternal separation (90, 91). In response to acute stress, maternal deprivation leads, however, to higher transcription of the C*rh* gene and rapid secretion of ACTH and glucocorticoids (87, 92–94).

Enhanced maternal care due to natural variation (95, 96) reduces C*rh* expression in the PVNh in the adult offspring, curtails hormonal responses to stress, and enhances hippocampal GR levels. Similarly, daily brief (15 min) separations from the mother during postnatal week 1 up to 3 promotes an adult phenotype characterized by a sensitization of the HPA axis with an attenuated stress response (33, 97) associated with reduced basal expression of Crh in the PVNh and increased hippocampal expression of the GR (98, 99). Hereby, the reduction of C*rh* expression in the PVNh precedes further changes at different levels of the HPA axis compatible with the idea that C*rh* mediates handling-evoked maternal care to adaptation of the HPA axis (99, 100).

Sustained early-life stress can also be generated by limiting the amount of nesting material which creates an enduring stressor for the dam. This leads to fragmented maternal care and imposes an additional stressor on the pups (101, 102). By the end of 1 week of postnatal stress (P9), Crh mRNA expression in the PVNh of the early-life stress group is significantly reduced compared to undisturbed controls (102). Reduced C*rh* expression may reflect

altered glucocorticoid negative feedback and/or increased Crh release concomitant with a failure of acute stress-induced Crh production.

Collectively, these exemplary studies in rodents illustrate that early trauma and stress can cause sustained changes in Crh expression which critically depend on the developmental stage of the limbic-HPA axis. Are then any of these changes in C*rh* expression related to epigenetic mechanisms such as DNA methylation?

EPIGENETIC PROGRAMING OF C*rh*

The fetus grows up in the womb protected by the placental barrier which shields from any adversity that might arise in the maternal environment. Severe stressors and strains can, however, compromise the placental barrier and expose the developing fetal brain to maternally derived substances, such as cytokines or stress hormones. The latter can trigger increased vulnerability to depression, anxiety, schizophrenia, and autism (103). In this respect, male but not female mice offspring exposed to chronic variable stress early in gestation (embryonic day 1–7) displayed impaired behavioral stress responsivity, anhedonia, and an increased sensitivity to treatment with a selective serotonin reuptake inhibitor (104). Increased Crh expression in the amygdala is associated with decreased promoter methylation (**Figure 4**), while decreased GR expression in the dorsal hippocampus correlated with increased methylation at the NGFI-A binding site of exon 1_7 (see below). Notably, the fetal brain was unformed at the time the stressor was applied to the mother indicating that sex-specific changes in fetal development and long-term adaptation of the HPA axis might be mediated through effects on the developing placenta. In this regard, male control placentas showed lower Dnmt1 expression compared to females. Moreover, increases in placental Dnmt1 in response to prenatal stress were largely confined to females. How increased Dnmt1 expression might counteract prenatal stress-dependent perturbations in placental gene expression remains presently unknown.

In a postnatal study, rats exposed to early-life stress (maternal separation) were studied for HPA-axis responses to acute restraint stress and C*rh* promoter methylation in the PVNh nucleus and the central nucleus of the amygdala (105). Despite slightly lower plasma ACTH, plasma corticosterone levels in female rats subjected to early-life stress were significantly higher in basal conditions and after exposure to acute restraint stress. In males, early-life stress did not affect basal plasma corticosterone levels, but in females the increases after restraint were significantly higher in early-life stressed males than in controls. In accord with these findings, Crh mRNA expression in the PVNh was increased under resting and acute stress conditions in females while increases in males occurred only under acute stress conditions (**Figure 4**). Moreover, Crh expression in the central amygdala was unaffected in either condition in both sexes. Early-life stress associated with hypomethylation of the C*rh* promoter in the PVNh in either sex and mapped to two CpG residues localized next to and at the dyad symmetry axis of a cyclic AMP responsive element (CRE). Methylation of the latter CpG residue impaired DNA binding of the activated form of the CRE binding protein (p-CREB) compatible with the idea that early-life stressed induced hypomethylation

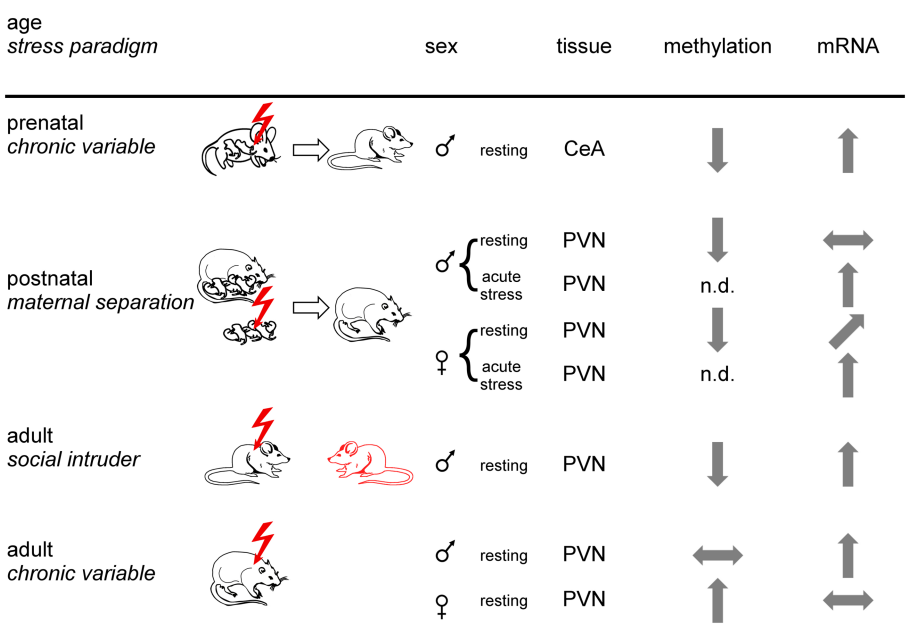

FIGURE 4 | Experience-dependent epigenetic programing of C*rh*. Prenatal, postnatal, and adult mice or rats were exposed to different stress paradigms (chronic variable, maternal separation, or social intruder stress). Sexes are indicated by respective symbols. Results from C*rh* promoter methylation and expression are depicted for the paraventricular nucleus (PVN) and the central amygdala (CeA). Tissues were isolated under resting conditions or following application of an acute stressor. Arrows show increased, decreased, and unaltered CpG methylation and Crh mRNA expression, respectively, for the indicated brain regions. Non-determined (n.d.) refers to the absence of information. In sum, epigenetic programing of C*rh* occurs in a stressor-, sex-, and tissue-specific manner.

facilitates CREB binding and subsequently basal and activated Crh transcription.

Exposure to social defeat over 10 consecutive days imposes sustained social stress on adult male mice resulting in anhedonia and social avoidance. This well-known paradigm associated with demethylation at the proximal C*rh* promoter (106). As a result, Crh mRNA expression in the PVNh was significantly increased (**Figure 4**). Interestingly, Crh demethylation was confined to males displaying signs of stress while those being unaffected escaped demethylation and Crh overexpression. Given that genetically homogenous mice were housed and tested under standardized operating procedures this differential response suggests that subtle differences in the previous life histories can modify the outcome from exposure to stress. Application of imipramine over 3 weeks, a clinically relevant time period, improved the social avoidance behavior and reversed changes in C*rh* promoter methylation and gene expression. C*rh* demethylation was preceded by decreases in Dnmt3b and Hdac2 expression, while Gadd45b expression was temporarily increased. In this regard, Gadd45b has been shown to subserve demethylation by facilitating recruitment of the DNA repair machinery (107, 108).

In a further study, adult male and female rats were exposed to chronic variable stress after which C*rh* expression and promoter methylation was measured in the PVNh, the bed nucleus of the stria terminalis and the central amygdala (109). Chronic variable stress produced C*rh* promoter hypermethylation at specific CpG dinucleotides in all tissues examined in females while the effects in males were confined to the stria terminalis and the central amygdala. In the PVNh, chronic variable stress increased Crh expression in the males (in the absence of changes in methylation), while the Crh peptide decreased in females most likely due to increased promoter methylation (**Figure 4**). This study firstly evidenced sex-specific epigenetic programing of C*rh* and illustrates the importance of sex differences in brain epigenetics (110). The functional implications of decreased Crh expression in females in the presence of elevated corticosterone levels requires, however, further investigations.

Together, these studies show that epigenetic programing of *Crh* can occur in a stressor-, tissue-, and sex-specific manner. Moreover, differences in the previous life history might protect from epigenetic programing. Subsequent variations in DNA methylation at the C*rh* gene can serve to distinguish the transcriptional response to later stress-related stimuli. Experience-dependent programing of gene expression potential can thus confer an increased risk to anewed stress exposure and the manifestation of stress-related diseases such as depression and PTSD (**Figure 5**).

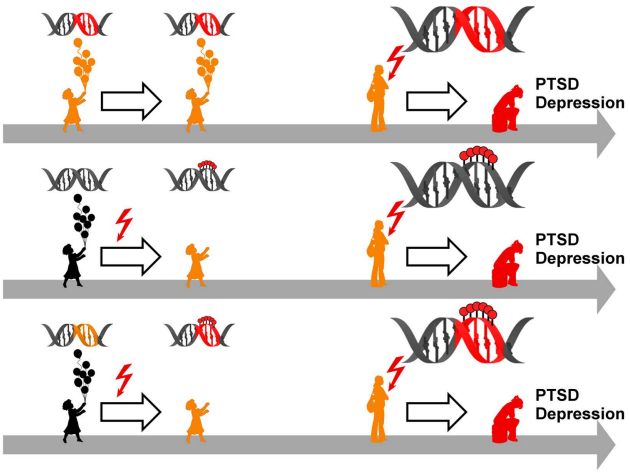

FIGURE 5 | Model for epigenetic risk factors in PTSD and depression. Genetic mutations (red segment of the DNA double helix) can confer an increased vulnerability to PTSD and depression which manifest following anew exposure to stressful life events (red flash). Although carrying a predisposition to disease (symbolized by orange colored child and adolescent), such individuals can stay healthy in the absence of trauma and stress (upper panel). Early life adversity (red flash) can elicit epigenetic programing of stress genes via DNA (de-)methylation (symbolized by red lollipops on DNA double helix) leading to altered expression. These alterations confer an increased vulnerability to later on trauma and stress and ultimately result in manifestation of PTSD and depression (middle panel). Genetic mutations (orange segment of the DNA double helix) can serve as a substrate for epigenetic programing in response to early trauma and stress (red flash) via DNA (de-)methylation (symbolized by red lollipops on red segment of DNA double helix). This pre-activation can result in PTSD and depression following anew exposure to trauma and stress (lower panel).

EXPERIENCE-DEPENDENT PROGRAMING OF Avp

Newborn rats exposed to repeated brief maternal separation and handling (PND 1–10) showed as adults reduced social investigative interactions and increased aggressive behavior with a higher frequency of attacks in the social interaction test. The latter behavior was detected only in males which displayed an increased number of Avp-positive magnocellular neurons in the PVN (111).

A related phenotype was detected following maternal separation in mice (112). While both sexes displayed increased anxiety-related behavior (reduced time spent on open arms in the elevated plus-maze and reduced exploration time of novel objects) this alteration associated with sex-specific differences in Avp expression. In this respect male but not female mice showed increases in Avp immunoreactivity in magnocellular neurons of the PVN and the lateral hypothalamus.

Sex differences in response to early-life adversity are not limited to Avp but extend to the receptor level. Adult rats that were reared by high licking and grooming mothers showed increased Avp V1a receptor binding in the central nucleus of the amygdala (113).

A recent study in human strengthens the importance of sex differences in AVP expression and suggests possible therapeutic consequences (114). PTSD is associated with problems in intimate relationships, partly due to impaired social cognition. Attention to partner expressions of anger was investigated as an indicator of distress and need for affiliative behaviors to repair the relationship bond. AVP administration enhanced men's attentional engagement with their partners' expressions of anger and alleviated the negative impact of PTSD on this social cognitive process. Moreover, men's morning urinary AVP levels were negatively correlated with their PTSD severity. In contrast, no such effects were detected among women or for attention to unfamiliar men's or women's anger expressions.

Collectively, early-life adversity preferentially programs the expression of hypothalamic Avp and its receptor in male rodents. Moreover, deficits in social cognition in male PTSD patients are improved following AVP administration.

EPIGENETIC PROGRAMING OF *Avp*

Maternal separation (3 h per day from postnatal day P 1–10) has been used to study early-life stress in male mice. Consistent with previous studies, adult mice displayed a hyperactive HPA axis characterized by corticosterone hypersecretion under resting conditions and following application of an acute stressor. These findings associated with enhanced immobility in the forced swim test and memory deficits in an inhibitory avoidance task (115).

Whereas hypothalamic Crh mRNA expression was not affected by early-life stress, the paradigm resulted in a sustained upregulation of Avp. This response was confined to the PVNh coordinating the stress response whereas Avp expression in the supraoptic hypothalamic nucleus which controls fluid homeostasis remained unaffected. Increased Avp expression correlated with reduced levels of DNA methylation at multiple CpG residues at a downstream enhancer region which serves to fine-tune gene expression. Hypomethylation evolved gradually in response to maternal separation to reach a maximum at 6 weeks. Thereafter, changes in DNA methylation declined slowly but were still detectable after 1 year (115).

Hypomethylation localized to high affinity DNA-binding sites for the methyl-CpG-binding protein Mecp2. This epigenetic reader serves as a scaffold for the assembly of DNMTs and histone deacetylases which can rewrite epigenetic marks to induce gene silencing (86, 116). Hypomethylation in 6-week-old early-life stress treated mice reduced Mecp2 binding at the *Avp* enhancer and consequently promoted increased gene expression.

Early-life stress treated mice showed, however, no signs of hypomethylation directly after termination of the stressor (postnatal day 10) although Avp expression was robustly increased. This finding indicated that stress signals unrelated to DNA methylation appear to control binding of Mecp2 at earlier stages. In support of this concept, depolarization-dependent Ca^{2+} influx and activation of calmodulin kinases has been shown to cause Mecp2 phosphorylation. This event impairs Mecp2 DNA binding and derepresses target genes (115). In accord with this hypothesis, early-life stress treated mice showed increased Mecp2 phosphorylation in parvocellular Avp-expressing neurons at postnatal day 10.

Taken together, these results suggest a stepwise mode for epigenetic programing of *Avp*. Initially, early-life stress leads to phosphorylation of the epigenetic reader Mecp2. This modification favors dissociation from the *Avp* enhancer and promotes increased expression. Moreover, due the absence of Mecp2, epigenetic writers like DNMTs and histone deacetylases loose their docking sites and will fall short to maintain DNA methylation. As a result *Avp* hypomethylation will gradually evolve and leave a persistent memory trace of the early-stress event encoding an altered stress regulation.

This stepwise mode of epigenetic programing of Avp in response to early trauma involves the transition from stress-induced posttranslational modifications (so called soft-wiring) to stable DNA modifications (so called hard wiring). Although there are so far no related studies in humans, we want to point out that changes following from exposure to early life adversity appear initially reversible. They might, however, solidify over time or upon re-exposure to stress. In this respect, early interventions such as improvements in parenting and the social environment might delay or protect individuals exposed to early trauma to develop disease at later stages.

EXPERIENCE-DEPENDENT PROGRAMING OF GR

Experience-dependent programing of the HPA axis also applies to negative feedback regulators like the GR. Enhanced maternal care during the first 10 days of life (95) and daily short (15 min) periods of maternal separation during the first postnatal week (98, 99) increase hippocampal GR expression in adult rodents. This event leads to decreased hypothalamic Crh expression and an attenuated response upon acute stress exposure over life span (117, 118).

On the other hand, extended periods of maternal separation (up to 4.5 h per day) change the direction of GR expression later in life and manifest with reduced expression of hippocampal GR in rodents. Consequently, negative feedback regulation of the HPA axis is impaired as evidenced by elevated levels of ACTH and corticosterone in adulthood (98). These early life dependent alterations in GR expression can be reversed by housing up-growing animals under an enriched environment which normalizes GR expression, behavioral, and memory performance (119).

EPIGENETIC PROGRAMING OF *GR*

The molecular foundations of epigenetic programing of the *GR* in response to early life care have been discovered by Meaney and Szyf in a series of elegant studies (120) [reviewed in (42, 121)]. Briefly, natural variations in maternal care can stimulate to different degree serotonin turnover in the hippocampus of 6-day-old rat pups, where GR expression is site-specifically increased. Following binding to its receptor, serotonin activates via protein kinase A the expression of the immediate early gene *NGFI-A* encoding a zinc finger transcription factor. NGFI-A protein binds in turn to its recognition site at exon 1_7 of the proximal *GR* promoter to enhance transcription.

How do these events establish a lasting record of maternal care? Multiple untranslated first exons of the proximal *GR* promoter are transcribed as a result of the associated promoter activity and are spliced on the common second exon to give rise to the same GR protein. The major part of the proximal *GR* promoter, including most alternative exons and the hippocampus-specific exon 1_7, are contained within a CGI. As alluded above, CGIs can be subject to DNA methylation and serve to control gene expression potential. In this respect the offspring from high-care taking mothers showed decreased CpG methylation at the NGFI-A binding site when compared to offspring of the same age from low-care taking mothers. These differences developed in the first postnatal week concurrent with differences in maternal care and its effect on NGFI-A expression (122). Differences in maternal care disappeared after the first postnatal week and consequently adult rats did not diverge anymore in hippocampal NGF-IA expression. Maintained hypomethylation left, however, an enduring memory trace of the postnatal environment and underpinned increased GR expression restraining HPA-axis activity in response to future stressors.

Epigenetic programing of *GR* occurs also in adult mice. Chronic and acute stress during adulthood induces changes in the methylation level of the GR promoter at exon 1_7 in the pituitary and adrenal gland whereas brain tissues remained unaffected (123). This result corroborates above findings from *Crh* and suggests that epigenetic programing is tissue-specific dependent on developmental stage.

The hypothesis that early life adversity in humans induces similarly epigenetic programing of hippocampal *GR* expression has been addressed by postmortem brain analysis from suicide victims (124). The documented histories of childhood adversities included severe sexual or physical abuse and neglect. Interestingly, total GR and exon 1F (the human homolog of rat exon 1_7) expression in the hippocampus was reduced in suicide victims with a history of abuse, but not in those without such a history or in control subjects. These results indicate that *GR* expression associated with early life adversity rather than with circumstances of life leading the way to suicide. These results agree with above findings from rodents that epigenetic programing of GR depends on the developmental context. Together, these studies in human and rat support the concept of a DNA-based memory that underpins the lasting effects of early life adversity.

Does then prenatal adversity also cause epigenetic programing of *GR*? Children whose mothers' were exposed to enduring stress during pregnancy (i.e., depression, anxiety, death of a relative, or marital discord) suffer from increased rates of psychological and behavioral disorders as adults (103). Hereby, the respective offspring frequently shows signs of HPA-axis deregulation similar to those detected in their depressed mothers (125). In support of above consideration, maternal depression in the third trimester led to an increased neonatal methylation of the presumed NGFI-A DNA-binding site situated at exon 1F in cord blood cells. Additionally, newborn methylation at nucleotide residue CpG3 associated with an enhanced cortisol response to an experimental stressor at 3 months of age (126). These data suggest that gestational depression can elicit epigenetic programing of peripheral and possibly central *GR* expression in neonates which might result in altered HPA-axis responsiveness in small children.

How long does epigenetic programing of neonate *GR* in response to maternal depression last? In a further study exon 1F methylation was measured in a group of children in the range from 10 to 19 years and in their respective mothers. Retrospective psychological evaluations were used to identify those mothers who had been exposed to physical or psychological abuse before, during, or after pregnancy. Exon 1F methylation was increased in adolescent children whose mothers' had experienced intimate partner violence during pregnancy (127). In contrast, adolescent exon 1F methylation was unaffected in case of prenatal or postnatal maternal abuse pointing to the critical role of gestational stress in epigenetic programing of fetal *GR*. Moreover, none of the respective mothers presented with altered exon 1F methylation in peripheral blood mononuclear cells (PBMC) suggesting that epigenetic programing of *GR* is confined to few sensitive time windows during development.

In further support of this idea, individuals suffering from major depression or borderline personality disorders exhibit increased exon 1F methylation in PBMC. Changes in DNA methylation were more prominent in the latter group and correlated in either with a history of child abuse, its severity, and the exposure to multiple types of maltreatment (128). Interestingly, healthy adults with a history of childhood adversity (maltreatment, poor quality parenting, loss of parents) show as well increased CpG3 methylation at exon 1F in PMBC and an attenuated cortisol response to the dexamethasone/CRH test (129). This finding corroborates that variation in parenting experiences in humans can bring about epigenetic programing of GR and HPA-axis deregulation without clinical manifestation. In this respect, additional stressors appear necessary to give rise to diseases such as depression and PTSD (**Figure 5**). Collectively, changes in exon 1F methylation seem to fulfill the criteria of an epigenetic risk marker to identify persons at future risk. Follow-up studies are needed to test this hypothesis and to explore exon 1F methylation as a potential biomarker for treatment response and relapse upon disease's manifestation.

GENE-ENVIRONMENT INTERACTIONS IN PTSD

Since the days of Garrod (130), medical research has been puzzled by the fact that the etiology of most common diseases depends not only on discrete genetic and environmental causes but also on the interaction between the two. Separate estimates on the contributions of genes and the environment to a disease without considering their interactions will only incorrectly describe the proportion of the disease (i.e., the population attributable risk) that is explained by genes, the environment, and their joint effects (**Figure 5**). A landmark discovery in this respect was the finding that the association of a polymorphism in the promoter region of the serotonin transporter gene (SLC6A4, commonly known as 5-HTT) with depression is influenced by stressful life events (131). Following on the concept of gene-environment interaction has been increasingly applied to quantitative epidemiological studies, although the nature of the mechanism(s) underpinning such interplay remained so far elusive. Two recent studies on gene-environment interactions in PTSD shed light on the potential contribution of epigenetic mechanisms as a mediator between risk alleles and early life events.

The neuropeptide PACAP (pituitary adenylate cyclase activating polypeptide) binds to the G-protein coupled receptor PAC1 (132) and is involved in the brain's response to stress. The PAC1 protein is encoded by the gene *ADCYAP1R1* that has been recently suggested to influence in a sex-specific manner whether an individual will develop PTSD (133). A cohort of urban primary-care patients who had been exposed to severe trauma (child maltreatment, a serious accident or attack with a weapon) showed a positive correlation between peripheral blood levels of PACAP and the extent of posttraumatic stress syndromes, but only in women. High PACAP values correlated with physiological measures of the acoustic startle reflex which has been previously associated with PTSD. Moreover, in a translational animal model the mRNA expression of PAC1 was increased in the amygdala of adult mice following classical fear conditioning and as a function of estrogen exposure.

Notably, a common genetic variant (rs267735) in *ADCYAP1R1* associated with PTSD. This finding was replicated in a second cohort of patients exposed to trauma, once again only in women (133). Differential DNA methylation of *ADCYAP1R1* associated

with PTSD symptoms and included the variant rs267735 which maps to a predicted estrogen response element. In support of a regulatory role of this genetic variant, postmortem analysis of female brains evidenced differential expression of PAC1 mRNA as function of the underlying genotype.

Together, these findings offer insight into the question of why women are more likely than men to develop PTSD and suggest that traumatic stress can leave an enduring memory trace on the epigenome by DNA methylation at regulatory polymorphic sites of genes that participate in the stress response. The regulatory site at *ADCYAP1R1* maps to an ERE which undergoes DNA methylation in response to early trauma and thus constitutes an epigenetic risk factor for the later development of PTSD in women.

Similarly, a recent study suggested that a polymorphism in the FK506 binding protein 5 gene (*FKBP5*) can serve as a substrate for early trauma-dependent epigenetic marking to increase the risk for PTSD (134). FKBP5 controls GR function by reducing ligand binding and translocation of the receptor complex to the nucleus (135). Hereby, FKBP5 forms part of a negative, intracellular, ultrashort feedback loop due to GR-dependent transactivation at a glucocorticoid response element (GRE) situated in intron 2. A polymorphism identified in this intron gave rise to a potential TATA element supporting the assembly of the transcriptional machinery. Adults who were homozygous or heterozygous for the A (risk) allele at this polymorphism rather than the G (protective) allele were more likely to suffer from PTSD – provided they had experienced abuse as children. The risk allele conferred an altered chromatin structure and increased transcription of *FKBP5*, hence strengthening the negative feedback loop that attenuated GR activity. Impaired GR function, in turn increases the hormonal response to stress.

How do traumatic experiences then act together with the risk allele in PTSD? Traumatic experiences lead to activation of the GR which is known to initiate changes in DNA methylation (136). Notably, CpG residues in intron 7 of FKBP5 – flanking a predicted GRE – were less methylated in risk allele carriers with a history of childhood abuse compared to those without a history of early trauma or traumatized patients without the risk allele.

In a translational *in vitro* model of early-life stress, treatment of hippocampal progenitor cells with dexamethasone elicited demethylation of intron 7 during proliferation and differentiation. The decline in methylation persisted over 20 days in steroid-free culture compatible with the idea of an enduring epigenetic mark. Furthermore, demethylation in intron 7 of *FKBP5* increased FKBP5 expression, GR resistance, and led to corresponding changes in the expression of downstream genes in PTSD patients harboring the risk allele. In contrast, risk carriers exposed to trauma in adulthood showed maintained DNA methylation at intron 7 suggesting that epigenetic programing of *FKBP5* is confined to a critical time window of hippocampal cell development. Compatible with this view, demethylation at intron 7 in hippocampal neuronal progenitor cells occurred only during the proliferative phase (134).

Consistent with the role of glucocorticoids in controlling Fkbp5 expression via DNA methylation, treatment of adolescent mice with corticosterone across 4 weeks enhanced gene expression in the hippocampus, hypothalamus, and peripheral blood cells (137, 138). This was accompanied by demethylation at intron 5 and 1 in brain tissues and peripheral blood cells, respectively. Interestingly, either intron includes a GRE binding side. Moreover, treatment doses correlated with Fkbp5 expression and demethylation at intron 1 indicating that intron 1 demethylation could serve as a biomarker for prior glucocorticoid load (138).

Together, these findings support the concept that exposure of children carrying the risk allele to stress can elicit enduring epigenetic changes in FKBP5 gene expression predisposing them to stress-associated disorders such as PTSD (**Figure 5**).

CONCLUSION

More than 100 years have passed since Sigmund Freud described the influence of early traumatic experiences on later mental health. Numerous epidemiological, clinical, and translational studies have corroborated the powerful role of early life adversity for the development of depression and PTSD. During sensitive windows of development, adversity can lead to enduring (mal-) programing of the stress system which constitutes an important risk factor for later disease.

Epigenetic mechanisms mediate the dialog between our genes and the environment and can elicit lasting changes in gene expression potential. Experience-dependent epigenetic programing of stress genes is increasingly recognized as an important pathway in the deregulation of stress systems in rodents and patients. Such programing can confer an enhanced risk on disease development upon re-exposure to trauma or stress (**Figure 5**). Epigenetic programing is, however, not deterministic. In translational models, an enriched environment can attenuate the effects of epigenetic programing. Epigenetic modifications appear amenable across all chapters in one's life and thus offer great opportunities for timely interventions comprising pharmaceutical and psychotherapeutic treatments.

Nearly twice as much women are affected by stress-related mood disorders compared to men. The HPA axis, the central mediator of the stress response, shows clear sex differences which seem to impact on the prevalence and course of stress-related diseases. Further clinical and translational studies focusing on sex differences in epigenetic programing of HPA-axis activity and brain functions in response to traumatic and social experiences can advance our insight into the sex-bias of PTSD and depression. Epigenetic biomarkers (i.e., *GR* and *FKBP5* in PBMS) in populations at risks appear promising to inform about previous stress exposure and require further clinical investigations.

With the decoding of the human genome at the turn of the millenium, medical research pinned its hope, above all, on the identification of gene variants coming along with an elevated risk of contracting a disease (**Figure 5**). However, it has become clear that genetic factors and environmental influences are not independent of each other and that acquired pieces of information deliver the instruction how to interpret the genetic blueprint. Whether or not a genetic predisposition manifests as PTSD or depression then seems to depend on how genes and environment conspire. Epigenetic programing in response to trauma or stress can be modified by genetic variation in the underlying substrates. Hereby, silent variations are switched into epigenetic risk

factors for PTSD or depression in response to additional adversities (**Figure 5**). Further studies are needed to address tissue-specificity of experience-dependent programing of genetic variants to distinguish between roles as biomarker, reflecting humoral changes in response to trauma versus roles as epigenetic risk factor driving disease development.

Taken together, epigenetic mechanisms play an important role in experience-dependent programing of stress genes and their genetic variants. Moreover, epigenetic biomarkers encoding previous trauma and stress history might guide early therapeutic interventions and risk management in PTSD and depression on a long-term perspective.

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How does a neuron "know" to modulate its epigenetic machinery in response to early-life environment/ experience?

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Exciting information is emerging about epigenetic mechanisms and their role in long-lasting changes of neuronal gene expression. Whereas these mechanisms are active throughout life, recent findings point to a critical window of early postnatal development during which neuronal gene expression may be persistently "re-programed" via epigenetic modifications. However, it remains unclear how the epigenetic machinery is modulated. Here we focus on an important example of early-life programing: the effect of sensory input from the mother on expression patterns of key stress-related genes in the developing brain. We focus on the lasting effects of this early-life experience on corticotropin-releasing hormone (CRH) gene expression in the hypothalamus, and describe recent work that integrates organism-wide signals with cellular signals that in turn impact epigenetic regulation. We describe the operational brain networks that convey sensory input to CRH-expressing cells, and highlight the resulting "re-wiring" of synaptic connectivity to these neurons. We then move from intercellular to intracellular mechanisms, speculating about the induction, and maintenance of lifelong CRH repression provoked by early-life experience. Elucidating such pathways is critical for understanding the enduring links between experience and gene expression. In the context of responses to stress, such mechanisms should contribute to vulnerability or resilience to post-traumatic stress disorder (PTSD) and other stress-related disorders.

Keywords: synapses, corticotropin-releasing hormone, CRF, glutamate, hypothalamus, epigenetics, stress, maternal care

INTRODUCTION

Neuronal gene expression is amenable to re-programing by environment and experience (1–3). The neuroendocrine stress axis is influenced by environment and experience during early postnatal development, and these changes endure. For example, maternal-derived sensory input is critical for setting the tone of the hypothalamus-pituitary-adrenal (HPA) axis for life via changes in the expression of glucocorticoid receptor (GR) in the hippocampus and of hypothalamic corticotropin-releasing hormone (CRH). High levels, or predictable bouts, of maternal-derived sensory stimulation result in an attenuated stress response and resilience to stress (4, 5). In contrast, early-life stress causes adults to exhibit augmented stress responses and cognitive impairments, associated with changes in expression of CRH and GR (6–8). Recently, it has been proposed that it is the *patterns* of maternal care that contribute crucially to the perception of stress early in life, and to the subsequent modulation of brain function. Thus, chaotic, fragmented sensory inputs from the mother influence neuronal networks involved in stress for the life of the animal in a direction opposite to that of predictable and consistent patterns (9). Thus, an important common basis may exist for both the beneficial and the adverse consequences of early-life experiences: the pattern of sensory input onto the developing brain might constitute an important parameter that influences the function of stress-sensitive neurons throughout life.

It is suspected that the endurance of the effects of sensory input during this critical period derives from activation of epigenetic mechanisms leading to changes in gene expression that are maintained throughout the lifetime. Here we review the neuroanatomical and molecular pathways bridging sensory input on a whole-brain scale with gene expression programing after distinct early-life experiences. We discuss the implications of these processes to post-traumatic stress disorder (PTSD).

EPIGENETICS AND EARLY-LIFE EXPERIENCE

The nature of epigenetic mechanisms is amply discussed throughout this collection of papers, and will not be described in detail here. Epigenetics offers an enticing explanation for how relatively brief sensory experiences may lead to long-lasting changes in neuronal function. Indeed, changes in components of chromatin, including DNA methylation or histone modifications have been examined after early-life experience, and found in several key genes involved in regulation of the HPA axis [GR, (10); CRH, (11); arginine vasopressin, (12)]. Here we focus on the lasting repression of CRH in hypothalamic neurons that results from positive maternal care early in life (13). This finding has

been confirmed by numerous subsequent studies (4, 5). We focus on the CRH gene both as an important regulator of the stress response (14) and as a likely contributor to the phenotype engendered by nurturing early-life maternal signals, because modulation of CRH function through blocking of CRH receptor type 1 recapitulated the effects of augmented maternal care in non-nurtured pups (15). A second reason for a focus on the CRH gene is its use as a "marker" gene: the reliable detection of CRH repression after augmented maternal care suggests that understanding the mechanism that represses CRH expression enduringly might provide a key to understanding general processes that influence expression programs involving numerous other genes as well. Finally, in the context of the current review, a significant body of literature has implicated aberrant expression and central (CSF) release of CRH in the pathophysiology of PTSD (16–19).

HOW DOES A CRH-EXPRESSING NEURON KNOW TO MODULATE CRH GENE EXPRESSION?

Corticotropin-releasing hormone gene expression is regulated by transcription factors, and these in turn are activated by signals that reach the nucleus from the membrane, and often involve calcium signaling (20). Synaptic input onto the CRH-expressing neuron includes a number of neurotransmitters, of which glutamate constitutes a major excitatory input (21). Indeed, glutamatergic signaling in the PVN is necessary for the initiation of the endocrine stress response, and glutamate receptor agonists delivered to the PVN drive CRH release (22, 23). Recent research has revealed that early-life augmented care leads to a transient reduction in the number and function of glutamatergic synapses to CRH neurons in the PVN (11). Using several methods (immunohistochemistry, electron microscopy, and electrophysiology), Korosi et al. discovered that (1) the number of glutamatergic terminals abutting CRH-positive neurons was reduced, (2) the number of asymmetric, putative excitatory terminal boutons onto CRH neurons was reduced, and (3) the frequency of spontaneous excitatory postsynaptic currents to PVN neurons was dramatically reduced (**Figure 1**). The same measures were taken in the thalamus and yielded no changes. Similarly, there were no changes in markers of inhibitory transmission. Together these data strongly support the notion that augmented maternal care reduces excitatory drive to the CRH-expressing neuron in the PVN.

Whereas the correlation between reduction in excitation and reduction of CRH expression is suggestive, it does not answer the question of causality: is reduced glutamatergic input to a CRH cell required and sufficient to repress CRH? To address this question, *in vitro* methods have been initiated, with the use of organotypic hypothalamic slice cultures to isolate the PVN. In this system, application of glutamate receptor antagonists (blocking both AMPA- and NMDA-type receptors) can effectively eliminate ionotropic glutamatergic transmission. Pilot data suggests that this manipulation may suffice to repress CRH mRNA levels compared to vehicle-treated controls (24). These initial findings are consistent with the notion that augmented maternal care reduces excitatory drive to the PVN, which in turn leads to reduced CRH mRNA production.

HOW DOES THE SENSORY SIGNAL FROM MATERNAL CARE REACH THE PVN AND SERVE TO REDUCE EXCITATORY SYNAPSE NUMBER AND FUNCTION?

Maternal input to her progeny consists of a variety of stimuli, among which sensory stimuli and especially touch (licking,

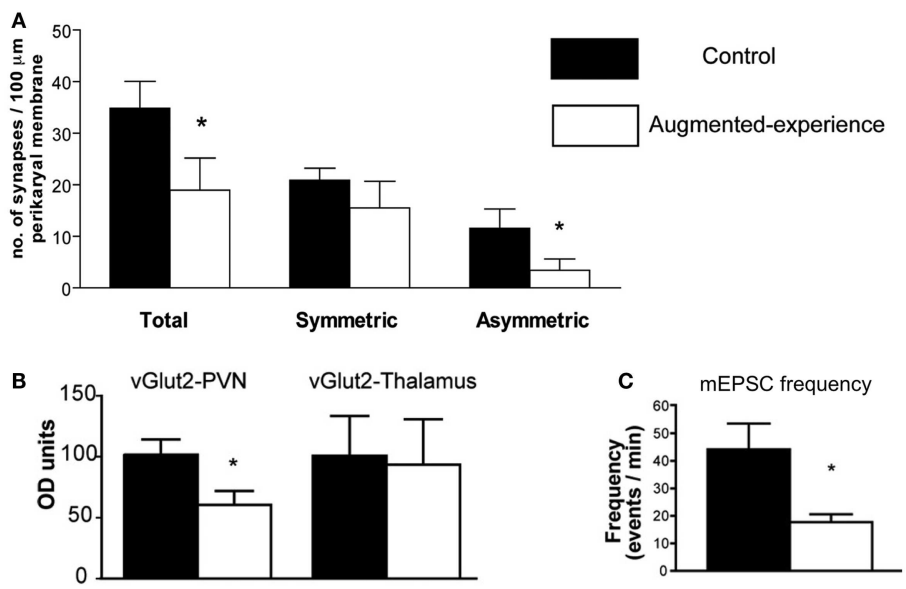

FIGURE 1 | Augmented early-life experience reduces the number and function of excitatory synapses in the paraventricular nucleus of the hypothalamus (PVN). **(A)** Total number of synapses was reduced by 50%, attributable to a 70% reduction of asymmetric (excitatory) synapses onto CRH-expressing neurons in the PVN. **(B)** Levels of the vesicular transporter vGlut2, a marker of glutamate-containing synaptic vesicles, were reduced by approximately 40% in rats with augmented early-life experience relative to controls. **(C)** Miniature excitatory postsynaptic currents (mEPSC) frequency was reduced by 60% in putative CRH neurons. Adapted from Ref. (11) with permission from the Journal of Neuroscience.

grooming) appear to be the most important (25–27). Levine's group demonstrated that augmented HPA responses to stress caused by 24 h maternal deprivation could be prevented by stroking the pups, highlighting the importance of tactile stimulation to normal development of HPA activity (28). Using brain-mapping methods, the pathways through which these signals reach the PVN have been identified (29).

Glutamate-specific retrograde tracing revealed that excitatory afferents terminating in the PVN originate in the paraventricular thalamus (PVT), lateral septum, bed nucleus of the stria terminalis (BNST), and amygdala (30). The BNST integrates and relays signals from the limbic forebrain and amygdala and provides both inhibitory and excitatory drive to the PVN. Specifically, posterior sub-regions inhibit stress-induced CRH expression in the PVN, whereas anterior regions facilitate it (31). The central nucleus of the amygdala (CeA), important for integration of autonomic inputs, facilitates CRH release from the PVN (**Figure 2**), likely via the BNST (32, 33).

Importantly, both the CeA and BNST are activated by maternal care. Handling rat pups evokes a burst of nurturing behavior (licking and grooming) by the dam upon the pups' return to the home cage. A single instance of handling results in c-fos activation in both BNST and CeA (29), yet did not influence CRH expression. In contrast, recurrent handling for a week, which led to repression of CRH expression, was associated with c-fos activation also within the PVT (29). This suggests that the contribution of the PVT to the overall circuit that conveys maternal signals to the CRH cells in the PVN is important to reduce the expression of the gene. The PVT has been shown to play an important role in stress memory and adaptation (34, 35). The PVT sends afferents to the PVN, and possesses bidirectional connections with the CeA and BNST (36). Considering that the majority of PVT output to the structures described above are excitatory, how might PVT activation result in repression of the PVN? Here, we speculate that activation of the PVT might excite BNST regions that are known to inhibit CRH expression in the PVN (**Figure 2**).

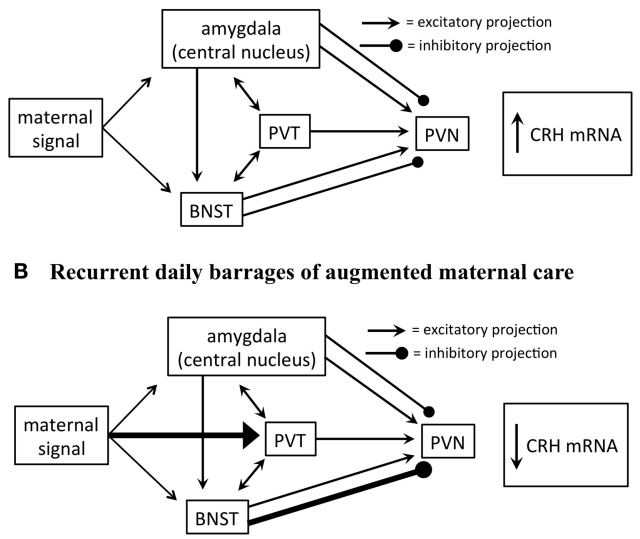

FIGURE 2 | Proposed circuitry involved in conveying maternal-derived sensory input to CRH-expressing neurons in the PVN. The PVN receives excitatory and inhibitory projections, including projections from the amygdala, paraventricular thalamic nucleus (PVT), and bed nucleus of the stria terminalis (BNST). These regions are also interconnected by excitatory projections (solid black lines). **(A)** The amygdala and BNST are both activated after a single day of handling-evoked augmented maternal care, and in turn stimulate the PVN (29). **(B)** The PVT is not activated after a single day of augmented maternally derived sensory input, but is recruited by recurrent daily barrages. This is thought to activate regions of the BNST that inhibit CRH-expressing neurons in the PVN (31). It is not fully known how this series of events promotes reduced numbers of excitatory synapses on CRH-expressing neurons.

INITIATION VS. MAINTENANCE OF EPIGENETIC REPRESSION OF CRH BY EARLY-LIFE EXPERIENCE

When considering the changes in gene expression that occur after augmented maternal care, it is important to note two key differences in timing. Repression of CRH begins around postnatal day 9 and persists through adulthood, while changes in glutamatergic signaling to the PVN were noted only at P9 and were back to control levels by P45 (11). This suggests that following the initiation signal mediated by reduction of glutamatergic signaling, there may be additional factors that are involved in maintaining the repression of gene expression that persists long past the initiating signal. Such factors are likely to be epigenetic in nature.

A likely suspect is the neuronal repressor neuron restrictive silencer factor (NRSF). NRSF is a transcription factor that silences gene expression via epigenetic modifications. The CRH intron contains a functional NRSF binding sequence (37), suggesting that the programing of the *crh* gene during early postnatal life may be due to NRSF activity. In fact, NRSF levels in the PVN are dramatically upregulated following augmented maternal care, starting at P9 and persisting into adulthood (11). This pattern is an inverse correlate of CRH expression levels following augmented maternal care, supporting the idea that NRSF may be involved in mediating CRH repression.

IMPLICATIONS FOR PTSD

Post-traumatic stress disorder is often associated with a history of early-life trauma (19, 38–40), and more specifically with chronic stressful situations such as abuse and long-lasting war rather than an acute event (41–47). PTSD is characterized by a persistently dysregulated stress response (19, 48, 49), and it is reasonable to assume that chronic early-life stressful events influences an individual's stress response to promote PTSD. There are several processes that might account for altered stress responses in PTSD. It has been posited that the hypothalamic-pituitary-adrenal axis is permanently sensitized by chronic early-life abuse, and this creates a vulnerability to subsequent trauma, resulting in PTSD. However, the mechanism of such sensitization is unclear. Here we provide a novel and plausible solution: if chronic early-life predictable and nurturing maternal care can reduce excitatory synaptic input onto stress-sensitive neurons in the hypothalamus, and hence "desensitize" future stress responses, then might abusive, erratic, or neglectful maternal behavior provoke the opposite? Augmentation of excitatory input to hypothalamic CRH cells may well serve to sensitize CRH release to future stresses. Whereas

this notion is speculative at this point, it is highly amenable to direct testing in animal models. A second possible basis of the abnormal stress response in PTSD that follows early-life chronic stress/abuse may include aberrant regulation of the expression of relevant genes, such as CRH. Here we provide insight into how early-life experience – nurturing or adverse – can result in persistently altered regulation of CRH expression. The lifelong changes in CRH release and expression that result from chronic early-life experiences may provide the neurobiological basis for resilience or vulnerability to subsequent stress, and hence to the development of PTSD.

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Epigenomic mechanisms of early adversity and HPA dysfunction: considerations for PTSD research

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Childhood adversity can have life-long consequences for the response to stressful events later in life. Abuse or severe neglect are well-known risk factors for post-traumatic stress disorder (PTSD), at least in part via changes in neural systems mediating the endocrine response to stress. Determining the biological signatures of risk for stress-related mental disorders such as PTSD is important for identifying homogenous subgroups and improving treatment options. This review will focus on epigenetic regulation in early life by adversity and parental care – prime mediators of offspring neurodevelopment – in order to address several questions: (1) what have studies of humans and analogous animal models taught us about molecular mechanisms underlying changes in stress-sensitive physiological systems in response to early life trauma? (2) What are the considerations for studies relating early adversity and PTSD risk, going forward? I will summarize studies in animals and humans that address the epigenetic response to early adversity in the brain and in peripheral tissues. In so doing, I will describe work on the glucocorticoid receptor and other well-characterized genes within the stress response pathway and then turn to genomic studies to illustrate the use of increasingly powerful high-throughput approaches to the study of epigenomic mechanisms.

Keywords: epigenetics, DNA methylation, early adversity, childhood abuse, brain development, hypothalamic-pituitary-adrenal axis, stress response, glucocorticoid receptor

INTRODUCTION

Childhood adversity can have life-long consequences for the response to stressful events later in life (1). Repeated exposure to trauma alters neurodevelopment (2), enhances the activity of endocrine mechanisms involved in the stress response (3, 4) and increases the risk of multiple forms of psychopathology (5, 6). For example, the risk of suicide is strongly linked to childhood sexual and physical abuse or severe neglect (7–9). Sexual and physical abuse or severe neglect in childhood are also well-known risk factors for adult forms of post-traumatic stress disorder (PTSD), at least in part via changes in neural systems mediating the endocrine response to stress (10). The hypothalamic-pituitary-adrenal (HPA) axis shapes the endocrine response to stress in addition to its role in many other physiological processes, including immune and metabolic function. As such, the HPA axis plays an adaptive role by maintaining allostasis (i.e., stability amid change) in the face of challenging environmental conditions. Part of the explanation for the enhanced impact of adversity in early life is thought to lie in the relatively high degree of plasticity during this period, when environmental factors exert pervasive effects on a number of health trajectories (11, 12). Accumulating evidence indicates that this phenomenon, sometimes called "biological embedding," involves persistent changes in gene regulation via epigenetic mechanisms (13). The goal of this review is to highlight research on epigenetic mechanisms of early life adversity and parental care – prime mediators of offspring neurodevelopment (11) – that addresses several critical issues for research in this rapidly evolving area. We conclude by providing examples of the ways in which research in this area may provide insights for PTSD researchers on the epigenetic impacts of early adversity and highlight challenges for the field going forward.

EPIGENETIC MECHANISMS: STABILITY AND CHANGE

A first critical issue in understanding the relative risk conferred by early life adversity concerns the molecular mechanisms mediating altered HPA function as well as other pathways underlying vulnerability that respond in a manner that is both contingent upon the adversity and stable in the face of similar perturbations in later life. Epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNA. The methylation of cytosine in cytosine-guanine dinucleotides (CpGs) in the DNA itself (i.e., 5meC) is the best understood epigenetic mark and the focus of the majority of current investigations. However other modifications to DNA, including hydroxymethylation (5-hmC) and other recently identified DNA modifications, are attracting increasing interest as potential gene regulatory mechanisms (14). It should be noted that the conventional methods used for mapping 5-mC, such as bisulfite sequencing and methylation-sensitive restriction enzyme-based approaches, do not differentiate it from 5-hmC. As such, although I use the term "DNA methylation" in this review to be consistent with the majority of primary publications to date, the term "DNA modification" is a more accurate descriptor. Variations

in these modifications occur as a result of genetic, stochastic, and environmental factors, all of which drive the epigenetic regulation of gene expression. There is some debate as to the primacy of stochastic and environmental factors in epigenetic variation (15). It is clear that proper epigenetic regulation is essential for normal development and cell division, conferring cell-type identity in a stable manner that appears to a large degree unresponsive to early life adversity. There also is now compelling evidence of epigenetic regulation by environmental factors. Epigenetic regulation thus provides a potential mechanism for understanding well-defined environmental effects on phenotypes.

Elucidating which regions of the genome are labile in response to early life adversity, how rapidly changes can occur, and the ontological time-course of epigenetic changes remains a matter of active investigation. As I discuss below, these epigenetic responses likely depend on the genomic loci under consideration. Humans are exposed to a variety of stressors throughout life, however early life stress appears to exert an profound effects on HPA function that is pervasive throughout life in part by altering epigenetic mechanisms in a stable manner. I will illustrate this point by discussing several studies in rodents that have provided foundational knowledge applicable to investigations in humans.

ANIMAL MODELS OF EPIGENETIC MECHANISMS IN EARLY LIFE SHAPING THE RESPONSE TO STRESS IN ADULTHOOD

Animal models of maternal care and perinatal stress have helped to provide a mechanistic understanding of the impacts of early life adversity, allowing for control of genetic variation and a temporal dynamics of environmental exposures. Classic examples are experiments pioneered by Levine in the late 1960s and Meaney beginning in the late 1980s that indicated that laboratory rodents exposed to different levels of maternal care show behavioral alterations in fearfulness in response to novel environments and endocrine-mediated stress responses (16). These studies have documented sustained alterations in the expression genes regulating HPA function, such as the Glucocorticoid Receptor (GR), in brain areas mediating anxiety behavior and HPA circuitry, such as the prefrontal cortex, hippocampus, and hypothalamus. As adults, the offspring of rat mothers providing relatively high or relatively low levels of maternal care display life-long alterations in DNA methylation and Histone 3 lysine 9 (H3K9) acetylation of the untranslated 1_7 splice variant of the GR promoter in the hippocampus and of the promoter of the GAD67 gene in the prefrontal cortex (17, 18). Other groups have provided evidence that additional genes in neural pathways mediating the stress response are epigenetically regulated in association with early life stress, including arginine vasopressin in the hypothalamus (19), and BDNF in the prefrontal cortex and hippocampus (20). Interestingly, apparently stable changes in GR promoter methylation emerge within the first week of life as a function of naturally occurring variations in maternal care. However, a recent study found evidence of sex-specific DNA methylation changes in BDNF and reelin in the medial prefrontal cortex of offspring subjected to an adverse maternal environment that emerge in the transition between adolescence and adulthood (21). These data indicate a complex temporal relationship between environmental adversity and epigenetic variation in the medial prefrontal cortex, dependent upon unknown mediating factors. The data suggest that the temporal dynamics of the epigenetic response to early adversity may, at least to some extent, be loci- and tissue-specific.

HUMAN STUDIES OF EPIGENETIC MECHANISMS IN EARLY LIFE SHAPING THE RESPONSE TO STRESS IN ADULTHOOD

In light of findings in animal models, GR is an obvious candidate gene of interest in exploring the relationship between epigenetic regulation as a function of early life adversity and mental health outcomes in humans. Perhaps less clear is the choice of appropriate cohorts and cell types in humans to test these relationships. As mentioned, epigenetic mechanisms play an important role in conferring cell-type identity during development and cell division. As a result, it is perhaps reasonable to assume that the impact of environmental factors on epigenetic marks is likely to be to some extent cell-type specific, limiting analysis to appropriate tissues of interest. We used hippocampal samples from suicide completers with and without a history of childhood abuse, and examined DNA methylation of the $GR1_F$ promoter, a region highly syntenic with the rat $GR1_7$ splice variant. We found higher levels of DNA methylation of the GR promoter region among suicide victims with a history of abuse or severe neglect in childhood, but not among suicide victims who were not abused in childhood or among a control group who had died of causes unrelated to suicide (22). This hypermethylation was associated with increased transcript abundance of both $GR1_F$ splice variant and total abundance of GR transcript, and *in vitro* analysis indicated that regions hypermethyated in abused suicide victims inhibited the binding of the EGR1 transcription factor (also known as NGFI-A, Zif268, Krox24, and ZENK) to select nucleotides within the promoter. Another recent study has replicated the finding of enhanced DNA methylation at this splice variant and gone on to identify altered DNA methylation in additional splice variants of the GR promoter and show that this response to early adversity is brain region specific, not occurring in the anterior cingulate (23).

STUDYING EPIGENETIC MECHANISMS OF HPA REGULATION BY EARLY ADVERSITY IN PERIPHERAL TISSUES IN HUMANS

A second important consideration for studies of the epigenetic response to early life adversity in living humans is its impacts on peripheral tissues, essential for efforts to sample potential changes over time and after interventions in humans. Lymphocytes are well-known targets of glucocorticoids, and immune profiles are known to be sensitive to alterations in GR abundance (24). One study found that childhood adversity (as measured by parental loss, childhood maltreatment, and parental care) was associated with increased DNA methylation of several sites within the GR1F promoter region in lymphocytes in adulthood (25). These results and other analogous data are important because they indicate that epigenetic alterations as a result of childhood adversity persist in peripheral tissues and are detectable in mixed lymphocyte cell populations. A recent investigation in whole blood of FKBP5, a negative regulator of GR, links PTSD to both genetic variation and early adversity (26). The authors of this study had previously characterized several genetic polymorphisms associated with PTSD risk. In the recent study, they found evidence of DNA demethylation in an intronic region only in individuals subjected to abuse

in childhood and only in those carrying the "risk" allele of the gene, with experiments in cultured cells indicating an effect shown to occur before and persist after differentiation in cultured hippocampal cells. In light of previous animal work showing that glucocorticoid exposure can drive DNA demethylation in mouse hippocampal dentate gyrus, indicating neural target tissues and *in vivo* conditions where glucocorticoid activity may modulate other HPA-responsive genes (27). These data investigating candidate genes demonstrate the capacity of the epigenetic machinery to respond to the psychosocial environment in early life in a manner that confers stable changes in stress pathways in lymphocytes – cells that evidently go through numerous cycles of cell division throughout life.

EPIGENOMIC REGULATION BY EARLY LIFE ADVERSITY IN GENE REGULATORY ELEMENTS AND BEYOND

A third consideration addressed by these studies is the need to identify genomic loci that are epigenetically labile in response to early life adversity. Studies to date have predominantly focused on epigenetic changes in gene regulatory elements (e.g., promoters) and defined candidate genomic loci. A study using a microarray approach combined with methylated DNA immunoprecipitation to interrogate promoter regions in all known protein-coding genes found that evidence of hypo- and hypermethylation among hundreds of genes in hippocampi from suicide completers with a history of early life abuse compared to non-abused controls (28). This study identified novel candidate genes (e.g., ALS2; involved in small GTPase regulation) and enriched candidate pathways (e.g., neuroplasticity) that may be epigenetically regulated in response to early life abuse and suicide. Another study of whole blood using the Illumina 450 K array, which examines the methylation status at single-nucleotide resolution in ∼480,000 CpG sites, covering most known genes and regulatory elements, found evidence of predominantly hypermethylated DNA within exons and 3′ UTRs of differentially expressed genes in PTSD patients with a history of early abuse, with epigenetic differences showing general agreement with levels of transcription (29). This study indicated that changes in DNA methylation among PTSD patients were enhanced in a with a positive history of childhood abuse, suggesting a potentially distinct epigenetic profile in this subgroup.

We documented changes in DNA methylation, H3K9 acteylation and gene expression across a 7 Mb region flanking the GR gene hippocampus using a tiling microarray approach in rats (30). Differences in the amount of maternal care received during the first week of life were associated with epigenetic differences over large genomic regions (∼100 kB) in hippocampi of adult animals. Differences in transcription occurred in the context of hyperacetylation and hypomethylation of promoters and hypermethylation of exons. Interestingly, hypermethylation within exons was the largest detect difference in DNA methylation as a response to higher levels of maternal care. Using this methodology, we identified a novel linkage between altered epigenetic status of a large protocadherin (PCDH) gene cluster of cell-adhesion molecules and maternal care. Previous studies have indicated that PCDH gene clusters regulate neuronal morphology and synaptic plasticity (31). It remains to be determined whether epigenetic alterations in these genes are linked to differences in neuroplasticity observed as a function of differences in maternal care (32). Nevertheless, as technologies for generating genome-wide epigenetic profiles become economically accessible to a wider array of researchers and bioinformatics tools for genomic analysis become more standardized, these approaches will likely provide powerful methods for hypothesis generation by consolidating multiple levels of biological information.

In a follow-up to this study, we analyzed the GR locus in hippocampi of adult suicide victims who were abused early in life compared to non-abused controls (33). Abused suicide victims showed broad statistical dependencies in DNA methylation differences in a manner akin to what was observed in the rat study described above (30). As in the previous study, the clustered PCDH gene cluster showed the largest alterations in DNA methylation within the locus examined. In humans, alterations in PCDH genes impair intellectual function, and mutations in PCDH genes are linked to autism (34). PCDH genes show evidence of distinct DNA methylation in whole blood from individuals with a childhood history of low socio-economic (35). The function of these epigenetic differences in PCDH remains unknown, however the data suggest that these genes are epigenetically labile in response to the early life social environment in both rodents and humans (33). Taken together, the data suggest that animal model of parental care may have broad applicability for understanding the consequences of epigenetic modification of PCDH gene pathways in humans.

An important caveat of these studies is that they often report data from mixed cell populations, potentially masking epigenetic differences in select cell types or skewing group differences due to cell admixture. Fluorescence-associated cell sorting followed by cell-type-specific epigenomic analysis is a potential solution. However, the relevant cell types are not often known, and cell types that are routinely extracted (e.g., $CD4^+$ T-cells) can often be divided into functional classes that are dissociable by additional rounds of selection, making it difficult to know whether one has attained the necessary level of specificity. An additional method to address this problem is informatic. Data gathered by the Encyclopedia of DNA Elements (ENCODE) project and other large-scale genomics initiatives are providing multidimensional representations of epigenetic and functional genomic signatures from a large number of cell types (36). These data will serve as important information on regions that identify cell types that can be used to bioinformatically deconvelute the constituents of cell admixture in mixed tissue populations [e.g., peripheral blood; (37)]. The data will also provide a valuable method to identify epigenetically invariant genomic regions that can serve to reduce genomic complexity in genome-wide analysis of epigenetic signaling, and transcriptional "silent" regions in specified cell types unlikely to be responsive to environmental perturbations. These data, together with an accumulating array of published epigenomic analysis, should help move research on the impacts of early life adversity beyond candidate gene to "candidate pathway" and "candidate network" levels of analysis, which are finding utility in other areas of complex disease research [e.g., (38)].

PROSPECTIVE FOR PTSD RESEARCH

Early life trauma shapes resiliency to stress in later life and is a risk factor for the development of PTSD, itself characterized by a "transformational" change in the neurophysiological response to stress that occurs in some but not all individuals exposed to trauma (39). Inter-individual differences in PTSD susceptibility

are modulated at least to some extent by early life adversity inasmuch as both are associated with HPA axis alterations – at least in a subset of PTSD patients. Both early life trauma or severe neglect and PTSD are generally associated with lower basal circulating cortisol levels and an attenuated response to acute stress challenge (10). These results have been proposed to explain a paradox of PTSD: namely that HPA dysfunction observed in PTSD appears distinct from that observed in chronic stress or major depression, conditions associated with *elevated* levels of cortisol. Because PTSD and major depression co-occur ∼50% of the time, the results indicate a distinct profile of PTSD in patients with a past history of trauma or early life abuse (10). Likewise, not all who experience trauma develop PTSD. A few studies have identified epigenetic variation associated with PTSD [e.g., (40)], and patients with a history of early life adversity may show distinct epigenomic profiles (29). These contrasts have made it challenging to identify epigenetic mechanisms linking early adversity to PTSD risk, calling for a variety of approaches in appropriate animal models and human studies. The molecular and epigenetic mechanisms associated with PTSD with and without a history of early life adversity are beyond the scope of the present manuscript, however this topic has been the focus of a number of excellent reviews [e.g., (10, 41–43)] – including in this volume (44).

Questions that need to be addressed for a more complete understanding of the role of epigenetic mechanisms in conferring risk of PTSD via early life adversity, include: when, precisely, during development, do epigenetic changes related to early adversity emerge? In what contexts, genomic regions/pathways, and in cell types? These principles remain poorly understood. However, some interesting parallels have been identified between regions of the genome that are epigenetically responsive to psychosocial factors (e.g., maternal care) in rodents, and syntenic regions of the human genome that are epigenetically labile in conditions of early adversity [e.g., childhood abuse; (33)]. Studies in animal models have suggested that early life stress impairs neuroplasticity in brain regions such as the hippocampus and has a lasting impact on endocrine systems underlying the response to psychosocial stressors (45, 46).

Many animals, including rodents and humans, appear to have evolved to respond both to immediate threats to life and limb and to psychosocial stress associated with predation risk, including via the transfer of information about environmental conditions to the offspring via maternal factors. For example, a number of studies in wildlife ecology and comparative endocrinology over the past 20 years have indicated that the influence of predators on stress in free-living animals is long-lasting, resembling stress effects in laboratory animal models of PTSD (47). Response mechanisms mediating the adaptive processes responsible for this transmission implicate the HPA axis and pathways involved in neuroplasticity (48, 49). Epigenetic research in this area is in its infancy, but offers an important avenue to study the extent to which developmentally regulated epigenetic mechanisms and environmental stressors interact in the context in which they have evolved.

Elucidating the biological mechanisms underlying effects of early social experiences on later mental health is challenging in humans for reasons that include technical/analytic complexity and limited access to relevant biological material. New methods that offer the ability to examine DNA methylation at single-nucleotide resolution genome-wide are advancing rapidly and, in tandem, a vast array of analytical tools and statistical methods are now available to normalize known technical biases, visualize epigenetic modifications, and identify differences among subjects (50). Genome-wide changes with early adversity appear to occur in association with pathway or network-specific alterations of the epigenomic landscape. Thus, the selection of epigenetic modification(s) for study and identification of the impacted pathways, which rely on computationally predicted and biologically validated relationships, remain a challenge for future studies. The use of whole-genome screens to identify stable combinations of epigenetic modifications that distinguish cell- or tissue-specific functional effects may be useful in tissue-specific gene targeting of therapeutics while minimizing off-target effects (51). It may not be clear, however, which cell types are relevant to the question under study. Nevertheless, there is some indication that even buccal epithelial cells may index the response to early life adversity, though not via epigenetic changes in GR *per se* (52). Buccal cells share embryonic stem cell origin with neurons, and therefore may provide a valuable means of identifying the epigenetic signature of early life adversity in young children, where blood sampling is problematic. In addition, because changes in epigenetic patterns are often only measured at one time-point, the involvement of later life experiences in conferring epigenetic changes are difficult or impossible to rule out. Prospective research validating the use of peripheral markers of early life impacts (which can also be done in animal models) will offer critical insights into the dynamic nature of epigenetic regulation and its role as a mechanism for programing gene function in response to early life trauma.

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Sex-specific and strain-dependent effects of early life adversity on behavioral and epigenetic outcomes

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Early life adversity can have a significant long-term impact with implications for the emergence of psychopathology. Disruption to mother-infant interactions is a form of early life adversity that may, in particular, have profound programing effects on the developing brain. However, despite converging evidence from human and animal studies, the precise mechanistic pathways underlying adversity-associated neurobehavioral changes have yet to be elucidated. One approach to the study of mechanism is exploration of epigenetic changes associated with early life experience. In the current study, we examined the effects of postnatal maternal separation (MS) in mice and assessed the behavioral, brain gene expression, and epigenetic effects of this manipulation in offspring. Importantly, we included two different mouse strains (C57BL/6J and Balb/cJ) and both male and female offspring to determine strain- and/or sex-associated differential response to MS. We found both strain-specific and sex-dependent effects of MS in early adolescent offspring on measures of open-field exploration, sucrose preference, and social behavior. Analyses of cortical and hippocampal mRNA levels of the glucocorticoid receptor (*Nr3c1*) and brain-derived neurotrophic factor (*Bdnf*) genes revealed decreased hippocampal *Bdnf* expression in maternally separated C57BL/6J females and increased cortical *Bdnf* expression in maternally separated male and female Balb/cJ offspring. Analyses of *Nr3c1*and *Bdnf* (IV and IX) CpG methylation indicated increased hippocampal *Nr3c1* methylation in maternally separated C57BL/6J males and increased hippocampal *Bdnf* IX methylation in male and female maternally separated Balb/c mice. Overall, though effect sizes were modest, these findings suggest a complex interaction between early life adversity, genetic background, and sex in the determination of neurobehavioral and epigenetic outcomes that may account for differential vulnerability to later-life disorder.

Keywords: maternal separation, postnatal, brain, epigenetic, mice, strain differences, sex-dependent

INTRODUCTION

The experience of adversity in the early stages of development can have a profound impact on psychological and physical health. In humans, this phenomenon is illustrated in studies of prenatal exposure to stress and nutritional deprivation (1–4) as well as studies of postnatal neglect and abuse (5–7). Maternal exposure to famine during pregnancy has been found to predict increased risk of schizophrenia and antisocial personality disorder (8, 9) and a history of childhood neglect is associated with an increased risk of depressive disorders, drug abuse, and suicidality (6, 10). Importantly, these adverse experiences may not be deterministic in predicting later-life disorder, but instead generate a vulnerability to later-life stress or trauma. This model of disease etiology is perhaps best illustrated in the pathophysiology of post-traumatic stress disorder (PTSD). Risk of PTSD is significantly higher in individuals who have experienced early life stress (e.g., physical/sexual abuse, neglect) (11, 12) and individuals who experience early life stress are more likely to be exposed to trauma in later-life (13, 14). However, it is notable that only a relatively small percentage of individuals that experience early life trauma (approximately 25%) develop PTSD (15). Thus, understanding the factors that promote both risk and resilience to the effects of early life adversity is essential to further exploration of psychiatric dysfunction.

Though epidemiological and clinical studies have been informative regarding the consequences of exposure to prenatal and postnatal adversity, studies of the underlying biological mechanisms of these exposures have relied primarily on animal models. In primates and rodents, prolonged separations between mother and offspring have been used to model elements of childhood neglect/maltreatment and have provided experimental evidence for the emergence of neurobiological and behavioral abnormalities associated with this form of adversity (6). These studies have identified many changes, including altered hypothalamic-pituitary-adrenal (HPA) function (16, 17) and neuronal plasticity (18, 19), that are shaped by postnatal maternal separation (MS). More recently, epigenetic changes have been identified which may underlie these enduring physiological and neurobiological effects (20, 21). Epigenetic modifications, such as DNA methylation and post-translational histone modification, have been the increasing focus of efforts to determine the molecular pathways through which adversity becomes biologically embedded within the brain and other tissues (22). In humans, the experience of severe social

deprivation (i.e., institutionalization from birth) or childhood abuse has been associated with altered DNA methylation profiles (23, 24). Psychiatric dysfunction is likewise linked to epigenetic variation in target genes and brain regions that have previously been implicated in the pathophysiology of these disorders (25–27). However, when considering the link between adversity, neurobiological dysfunction, and disorder, these human studies are limited by reliance on peripheral tissues (such as blood lymphocytes) or on post-mortem brain tissue, which may not necessarily map onto etiologically relevant epigenetic variation in the developing brain. Thus, animal models will continue to be critical methodological approaches in furthering our understanding of environmentally induced molecular and neurobiological change.

In the current study, our aim was to both determine the behavioral, brain gene expression, and DNA methylation changes induced by postnatal MS in mice and to determine whether these effects varied dependent on offspring strain and sex. There are a wide range of mouse strains/genotypes available for experimental laboratory studies and the "strain differences" in behavior of these mice have been well documented (28–32). Moreover, there is increasing evidence for the differential response of different strains of mice to environmental variation (33, 34). This differential responsiveness to environmentally induced behavioral change may also manifest in differential neurobiological and epigenetic change (35–38). Here we determined the effect of postnatal MS on C57BL/6J (B6) and Balb/cJ (Balb/c) mice – two strains with highly divergent behavioral phenotypes, particularly on measures of social/maternal, anxiety-like, and depressive-like behaviors (31, 35, 39, 40). In addition, within both strains, we determined the impact of MS on both male and female offspring. Sex-dependent effects of adversity have been shown in studies of prenatal stress (41, 42), *in utero* toxin exposure (43, 44), and postnatal maltreatment/neglect (45) and there is a significant sex-bias in the prevalence of most forms of psychopathology (46). Thus, it is of critical importance to understand the interaction between sex and exposure to adversity at a neurobiological and molecular level of analysis to determine the pathways through which these sex-dependent effects emerge. Moreover, there is increasing evidence that sex differences in themselves are associated with epigenetic variation – likely due to both genetic and hormonal differences between males and females (47, 48). Mother-infant interactions during postnatal development may likewise induce sex differences and have sex-dependent effects (49). Our experimental approach, through incorporation of both sex and strain was hypothesized to identify key variables that contribute to risk or resilience to adversity-induced effects.

RESULTS

Study design is presented in **Figure 1**. The MS protocol (see Materials and Methods), involving prolonged, daily separation between dams and litters from postnatal days (PND) 1–14, was implemented in B6 and Balb/c mice and compared to a control rearing condition (standard laboratory rearing with no separation). From PND35 to PND 40, offspring were assessed on the following behavioral measures: open-field, sucrose preference, and social interaction. Following behavioral testing, in a subset of offspring, brains were dissected (prefrontal cortex and hippocampus) for analyses of gene expression and DNA methylation of the glucocorticoid receptor (*Nr3c1*) and brain-derived neurotrophic factor (*Bdnf*) genes. These gene targets were chosen as they have been previously demonstrated: (1) to be epigenetically regulated by DNA methylation (50, 51), (2) to exhibit plasticity in expression in response to a broad range of environmental exposures (23, 50, 52–54), and (3) to be within mechanistic pathways involved in HPA responsivity and neuroplasticity that have been implicated in the pathological psychiatric outcomes linked to the experience of adversity (55, 56).

MATERNAL SEPARATION EFFECTS ON OPEN-FIELD ACTIVITY AND EXPLORATION

The open-field test is a standard measure of response to a novel environment (57). Activity (total distance traveled within the field) and exploration (movement within the anxiogenic inner area of the field) in rodents have been shown to differentiate individuals based on the experience of early life adversity (58, 59). In B6 mice, we found a rearing condition by sex interaction [$F(1, 36) = 4.64$, $p < 0.05$] on total distance traveled during testing, such that MS-reared males exhibited increased activity levels compared to control-reared males, with no rearing effect in B6 females (**Figure 2A**). In contrast, MS had no effect on activity levels in Balb/c mice (**Figure 2B**). Latency to enter the inner/anxiogenic area of the open-field was not found to be altered by rearing condition in B6 mice (**Figure 2C**). In Balb/c mice, we found a significant sex-specific rearing condition effect on this measure, with MS-reared females exhibiting shorter latencies to enter the inner area compared to control-reared females [$\chi^2(1, 19) = 8.13$, $p < 0.01$;

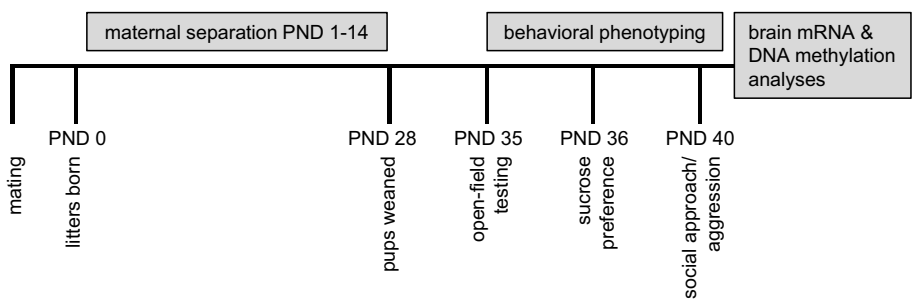

FIGURE 1 | Summary of experimental design.

Figure 2D]. Time spent in the inner area of the open-field, a typical measure of anxiety-like behavior (57), was not found to be altered by rearing condition in B6 or Balb/c mice (**Figures 2E,F**).

MATERNAL SEPARATION EFFECTS ON SUCROSE PREFERENCE

Preference for sucrose vs. water is used as a measure of reward sensitivity or hedonic motivation and in animal models of depression, a reduction in preference for sucrose is typically observed (60–62). Consistent with previous reports (63), we found Balb/c mice to have overall reduced sucrose preference compared to B6 mice.

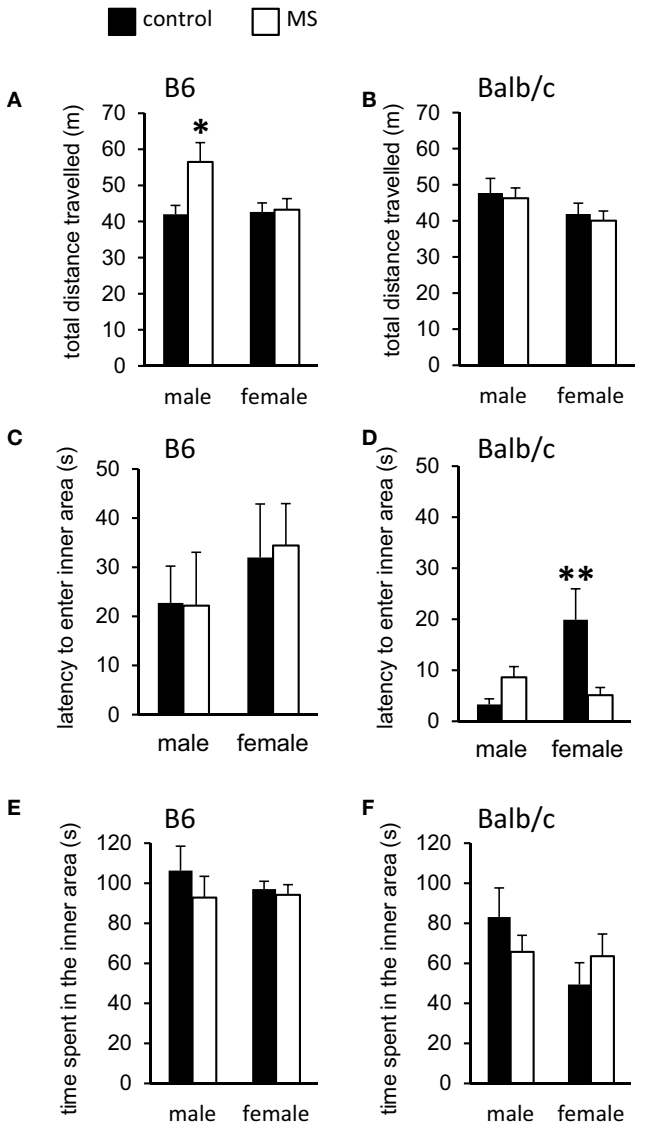

FIGURE 2 | Open-field behavioral effects of MS-rearing in B6 and Balb/c mice. Open-field activity (total distance traveled) was **(A)** increased in MS-reared B6 males ($p < 0.05$) with **(B)** no effects in Balb/c mice. Latency to enter the inner area of the open-field was **(C)** not altered by MS in B6 mice and **(D)** was decreased in MS-reared Balb/c females ($p < 0.01$). No effects of MS were observed on time spent in the inner area of the open-field in **(E)** B6 or **(F)** Balb/c mice. $*p < 0.05$, $**p < 0.01$ (control vs. MS comparisons).

All mice exhibited a higher than 50% average sucrose consumption (range 53–95%), indicating that the sucrose solution used was sufficiently rewarding and that no aversion to the sucrose solution was observed. We classified mice as having a preference for sucrose if they consumed more than 75% sucrose (as a percentage of total consumption) across the 3-day testing period. This definition of "preference" is consistent with previous studies of motivation in which the preferred stimulus must be favored 25% more than the comparison stimulus (64). Within B6 mice, both males and females that had experienced MS displayed reduced sucrose preference [males: $\chi^2(1, 18) = 2.38$, $p < 0.05$; females: $\chi^2(1, 19) = 2.22$, $p < 0.05$; **Table 1**]. Interestingly, within Balb/c mice, we observed sexual dimorphism in sucrose preference in control animals (males consumed more sucrose than females) that was reversed by MS; MS-reared males exhibited reduced sucrose preference whereas MS-reared females exhibited elevated sucrose preference [males: $\chi^2(1, 19) = 2.45$, $p < 0.05$; females: $\chi^2(1, 19) = 2.78$, $p < 0.05$; **Table 1**].

MATERNAL SEPARATION EFFECTS ON SOCIAL APPROACH AND AGGRESSION

Deficits in social behavior are a core feature in many forms of psychopathology (65) and impaired social interactions have been observed following exposure to reduced mother-infant interactions (66). Latency to sniff and aggressive behavior during dyadic social encounters with a novel stimulus mouse (129Sv strain) were assessed in control-reared vs. MS-reared mice. In B6 mice, we found a sex-specific rearing condition effect on latency to sniff the stimulus mouse, with shorter latencies observed amongst MS-reared B6 males [$\chi^2(1, 16) = 7.61$, $p < 0.05$] and no effect of rearing condition in B6 females (**Figure 3A**). No rearing condition effects were observed in Balb/c mice (**Figure 3B**). Across strains, aggressive behavior was only observed in males. Likelihood of displaying aggressive behavior was significantly increased in MS-reared Balb/c males (control: 66.7% vs. MS: 90%, $p < 0.05$) while this effect was not observed in B6 males (control: 30.5% vs. MS: 42.9%).

EFFECT OF MATERNAL SEPARATION ON CORTICAL AND HIPPOCAMPAL GENE EXPRESSION

Within the prefrontal cortex and hippocampus, we analyzed relative mRNA levels of *Nr3c1* and *Bdnf*. In B6 mice, MS was generally associated with a decrease in *Nr3c1* and *Bdnf*, though this effect was only statistically significant for *Bdnf* mRNA levels within the hippocampus (**Table 2**). Here we found a significant rearing condition

Table 1 | Percentage of mice exhibiting sucrose preference.

		Control %	MS %
B6	Male	63	**43***
	Female	56	**28***
Balb/c	Male	50	**20***
	Female	30	**60***

Statistically significant MS-induced effects are indicated in bold font; $*p < 0.05$ control vs. MS.

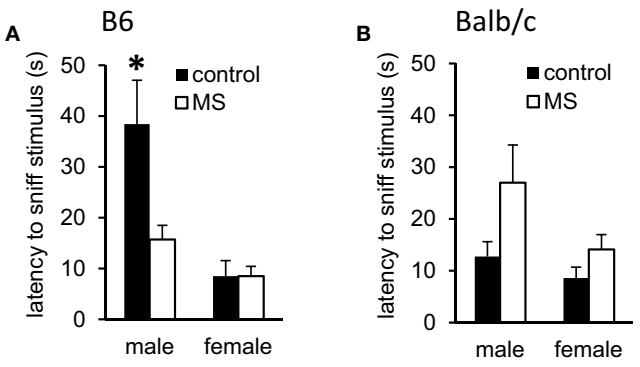

FIGURE 3 | Effects of MS-rearing on social behavior. Latency to sniff a novel mouse during dyadic social interactions was **(A)** decreased in MS-reared B6 males ($p < 0.01$) with no effect observed on this measure in **(B)** Balb/c mice. *$p < 0.05$ (control vs. MS comparisons).

by sex interaction [$F(1, 23) = 3.90$, $p < 0.05$], where B6 females that experienced MS had decreased *Bdnf* mRNA, with no rearing effect in males. In Balb/c mice, we found increased *Bdnf* mRNA in the prefrontal cortex of MS mice [both sexes; $F(1, 23) = 8.05$, $p < 0.01$; **Table 2**]. No other gene expression changes were noted in this mouse strain.

DNA METHYLATION CHANGES ASSOCIATED WITH MATERNAL SEPARATION

We analyzed DNA methylation across 8 CpG sites within the *Nr3c1* promoter region (see **Figure 4A**), which is highly homologous to the rat exon 1_7 GR promoter (50); this region also contains the binding site for the transcription factor NGFI-A (CpGs 7 and 8; **Figure 4A**). Analyses were conducted on average levels of DNA methylation across the 8 CpG sites to reduce multiple testing. In B6 mice, we found a significant rearing condition by sex interaction [$F(1, 23) = 3.85$, $p < 0.05$; **Figure 5A**], with elevated hippocampal CpG methylation in MS-reared males and no rearing effects in females. No rearing effects on GR methylation were detected in Balb/c mice (**Figure 5B**) or in the prefrontal cortex of B6 mice (**Figure 5A**). Within both strains, we found differences in CpG methylation associated with sex, such that in the prefrontal cortex there were elevated levels of methylation in females compared to males [B6: $F(1, 23) = 6.90$, $p < 0.05$; Balb/c: $F(1, 23) = 5.08$, $p < 0.05$]. Within the hippocampus, the converse was evident in Balb/c mice, with males having elevated DNA methylation levels compared to females [$F(1, 23) = 14.74$, $p < 0.01$; **Figure 5**].

We examined DNA methylation status of two regions of the *Bdnf* gene known to be epigenetically regulated: promoter region IV (51, 69) and promoter region IX (70) (see **Figure 4B**). Within the *Bdnf* IV promoter region, we analyzed DNA methylation across four CpG sites, including the CpG that lies within the binding site of the transcription factor CREB (CpG 1; **Figure 4B**). As with *Nr3c1*, analyses were conducted on average levels of DNA methylation across *Bdnf* CpG sites to reduce multiple testing. We found no rearing effects on DNA methylation (**Figures 5C,D**). Within the *Bdnf* IX promoter region (see **Figure 4B**), we analyzed DNA methylation across 5 CpGs. We found a rearing condition effect in the hippocampus of Balb/c mice, with increased DNA methylation associated with MS (both sexes) [$F(1, 23) = 4.82$, $p < 0.05$; **Figure 5F**]. No other rearing effects were determined (**Figures 5E,F**). Within promoter IX of the *Bdnf* gene, B6 males were found to have elevated hippocampal DNA methylation compared to females [$F(1, 23) = 51.43$, $p < 0.001$].

DISCUSSION

Our findings support the hypothesis that MS induces changes in behavior, brain gene expression, and DNA methylation in inbred mice. These findings also provide evidence for strain differences in response to MS and the interaction between sex and rearing experience in the prediction of these outcome measures. It does not appear to be the case that there is an overall "differential susceptibility" amongst B6 vs. Balb/c mice in their responsiveness to MS as there is evidence for MS-induced effects in both strains. However, strain responsiveness to MS does vary between measures, resulting in rearing effects in B6 mice on measures of open-field activity, sucrose preference, latency to approach a novel social stimulus, hippocampal *Bdnf* mRNA levels, and hippocampal *Nr3c1* DNA methylation. In contrast, rearing effects in Balb/c mice were observed on latency to enter the inner area of the open-field, sucrose preference, aggressive behavior toward a novel stimulus mouse, *Bdnf* mRNA levels in the prefrontal cortex, and DNA methylation of the *Bdnf IX* promoter region in the hippocampus. Even within the one measure that is altered in both mouse strains as a function of rearing environment – sucrose preference – the within-strain effect is different, with B6 males and females both exhibiting reduced sucrose preference and an interaction between sex and rearing condition in Balb/c mice (males showing decreased and females showing increased preference). Overall, these findings suggest that adversity experienced during postnatal development can manifest in divergent effects dependent on broad genetic characteristics, such as strain, and dependent on the sex of the individual experiencing the adversity; findings which point toward a very complex interplay between these individual- and group-level characteristics, the environment, and risk phenotypes.

EPIGENETIC EFFECTS OF ADVERSE ENVIRONMENTS

Though investigation of the effects of MS on behavioral and neurobiological outcomes is well established within the literature (16, 17), the incorporation of epigenetic analyses within these experimental designs is a relatively recent approach. In mice, MS-rearing has been previously demonstrated to induce hypomethylation of the vasopressin gene (*Avp*) within the hypothalamus leading to increased HPA reactivity amongst MS-reared offspring (20). Exposure to a single 24-h MS at PND9 has been associated with increased *Avp* DNA methylation in B6 mice and increased *Nr3c1* DNA methylation in DBA/2J mice (38). Similar to our findings, this study highlights the divergent epigenetic effects of MS in different mouse strains. Increased DNA methylation within the *Mecp2* (methyl CpG binding protein 2) and cannabinoid receptor-1 genes and decreased DNA methylation within the corticotropin releasing factor receptor 2 (*Crfr2*) gene has also been observed in the cortex of MS-reared B6 mice (21). Interestingly, these epigenetic changes were also observed in the sperm of MS-reared males

Table 2 | Relative mRNA levels of *Nr3c1* and *Bdnf* in the prefrontal cortex (PFC) and hippocampus (HIPP).

			Nr3c1		*Bdnf*	
			Control	**MS**	**Control**	**MS**
B6	PFC	Male	1.01 ± 0.06	0.84 ± 0.05	1.05 ± 0.12	1.00 ± 0.21
		Female	1.02 ± 0.11	0.98 ± 0.09	1.10 ± 0.12	0.86 ± 0.11
	HIPP	Male	1.02 ± 0.10	0.93 ± 0.06	1.04 ± 0.11	1.06 ± 0.13
		Female	0.96 ± 0.09	0.83 ± 0.09	1.03 ± 0.04	$\mathbf{0.66 \pm 0.07}$*
Balb/c	PFC	Male	1.01 ± 0.07	0.92 ± 0.08	1.04 ± 0.12	$\mathbf{1.29 \pm 0.10}$**
		Female	1.01 ± 0.07	1.05 ± 0.10	0.96 ± 0.09	$\mathbf{1.30 \pm 0.08}$**
	HIPP	Male	1.02 ± 0.08	1.03 ± 0.05	1.04 ± 0.10	1.32 ± 0.05
		Female	1.01 ± 0.10	0.83 ± 0.06	1.00 ± 0.09	1.00 ± 0.14

*Statistically significant MS-induced effects are indicated in bold font; *$p < 0.05$, **$p < 0.01$ (control vs. MS comparisons).*

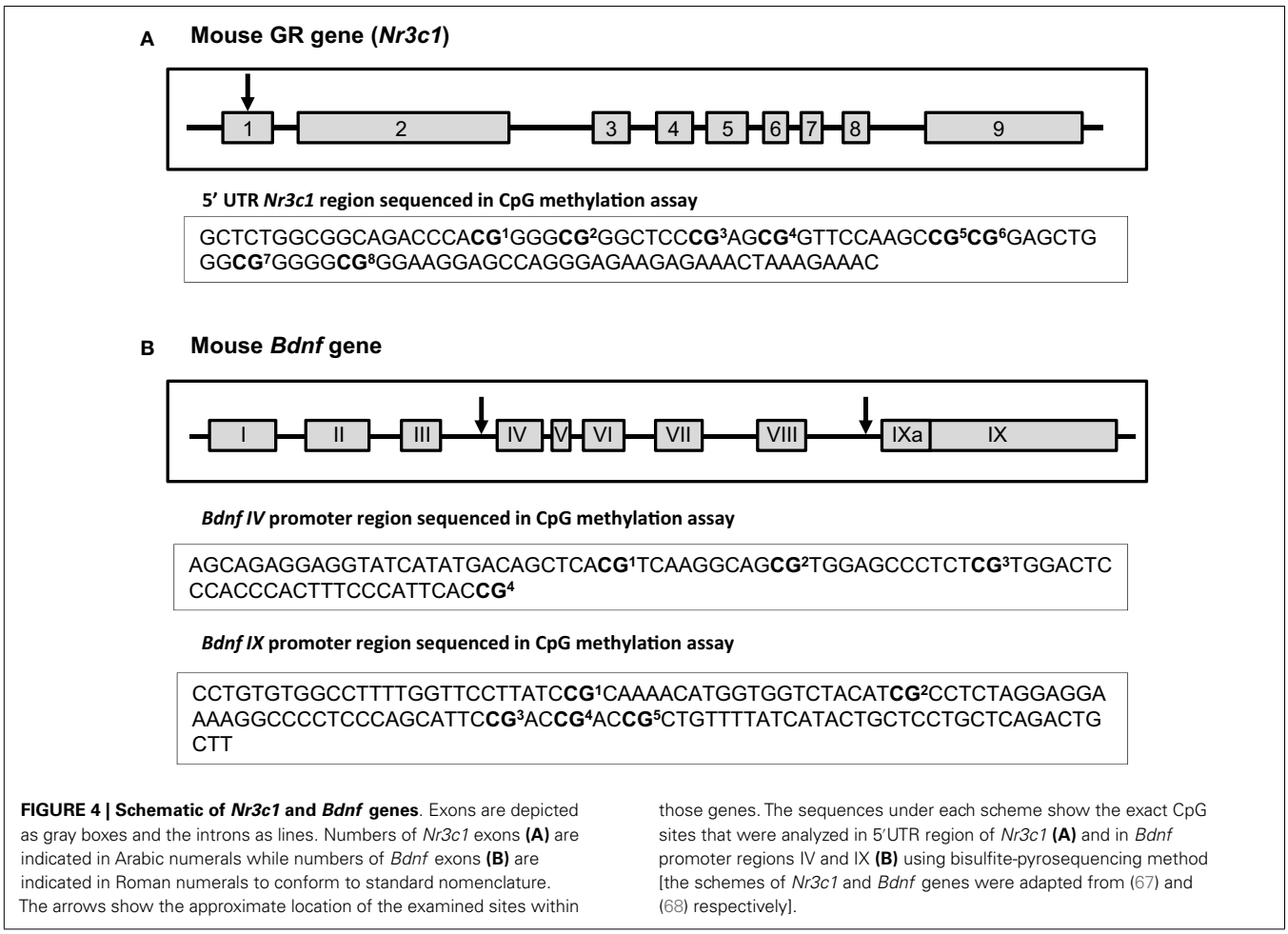

FIGURE 4 | Schematic of *Nr3c1* and *Bdnf* genes. Exons are depicted as gray boxes and the introns as lines. Numbers of *Nr3c1* exons **(A)** are indicated in Arabic numerals while numbers of *Bdnf* exons **(B)** are indicated in Roman numerals to conform to standard nomenclature. The arrows show the approximate location of the examined sites within those genes. The sequences under each scheme show the exact CpG sites that were analyzed in 5′UTR region of *Nr3c1* **(A)** and in *Bdnf* promoter regions IV and IX **(B)** using bisulfite-pyrosequencing method [the schemes of *Nr3c1* and *Bdnf* genes were adapted from (67) and (68) respectively].

and may account for the transmission of behavioral and epigenetic effects of MS-rearing across generations (21, 71). Beyond DNA methylation, there is also evidence for post-translational modification to histones associated with MS-rearing and pharmacological inhibition of histone deacetylases prior to MS can prevent the emergence of MS-associated risk phenotypes (72–74). Comparison of B6 and Balb/c mice on MS-induced histone changes suggests that altered cortical histone deacetylase mRNA (increased in juveniles and decreased in adults) is associated with MS-rearing in Balb/c but not B6 mice and that these enzymatic changes are associated with age-dependent differences in histone (H4) acetylation (73). This study suggests a biphasic epigenetic

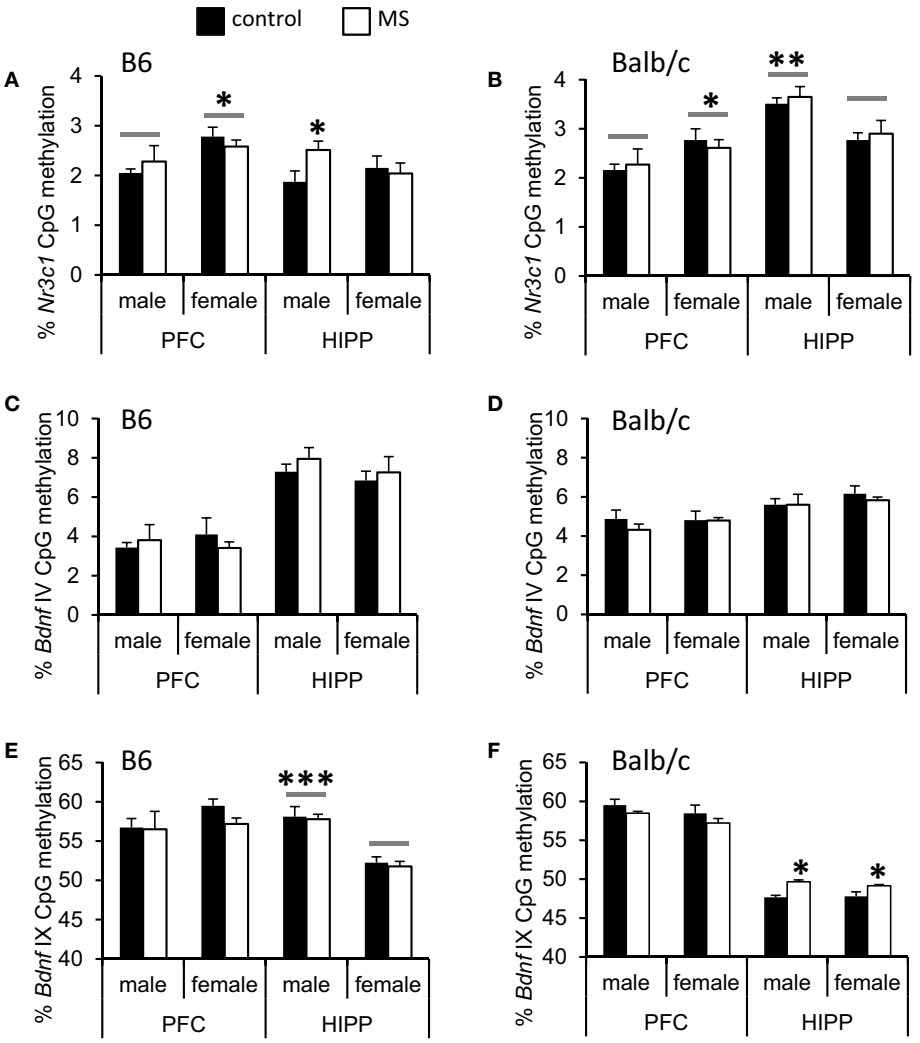

FIGURE 5 | Average percent DNA methylation of the *Nr3c1* and *Bdnf* promoter regions in the cortex (PFC) and hippocampus (HIPP). (A) Increased *Nr3c1* DNA methylation was observed in the HIPP of MS-reared B6 males and **(B)** no MS-rearing effects on DNA methylation of this gene in Balb/c mice. In the PFC, sex differences (indicated by a gray bar) were present in both B6 and Balb/c mice (elevated *Nr3c1* DNA methylation in females compared to males). In the hippocampus, Balb/c females had reduced *Nr3c1* DNA methylation compared to males. *Bdnf IV* promoter DNA methylation was not altered by MS-rearing in **(C)** B6 or **(D)** Balb/c mice. MS-rearing had **(E)** no effect on *Bdnf IX* promoter DNA methylation in B6 mice but **(F)** increased DNA methylation of this region in the HIPP of Balb/c mice. In B6 mice, females had reduced *Bdnf IX* promoter DNA methylation in the hippocampus compared to males (indicated by gray bars). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (control vs. MS comparisons or male vs. female comparisons).

response to adversity that may have consequences for the developmental timing of phenotypic (physiological, neurobiological, behavioral) outcomes associated with MS.

The epigenetic effects of MS contribute to a growing literature on the adverse effects of a broad range of early life experiences. In rodents, prenatal stress (42), nutrient deprivation (75, 76), variation in maternal care (50, 77), postnatal abuse (52), and post-weaning social environments (78) have been observed to induce epigenetic effects (DNA methylation and/or histone modifications). The *Nr3c1* and *Bdnf* genes examined in the current study appear to be highly plastic in expression and epigenetic regulation in response to these experiences (50, 52). These gene targets are also linked to the neurobiological pathways which may underlie risk of psychopathology. Glucocorticoid receptors within the hippocampus serve a critical negative-feedback role within the HPA axis such that elevated levels of these receptors are associated with an increased capacity to down-regulate the stress response and return to baseline glucocorticoid levels (55). Adverse early life experiences are typically associated with decreased *Nr3c1* expression levels and increased DNA methylation of the promoter region of this gene (23, 50). Though we did not find significant reductions in hippocampal *Nr3c1* expression, DNA methylation within the *Nr3c1* promoter was increased in MS-reared B6 males. *Bdnf* confers neuronal plasticity and has been demonstrated to alter mood and cognition (56, 79). Adverse early life experiences, such as abuse, have been demonstrated to decrease *Bdnf* expression and increase

Bdnf DNA methylation (52). Our data are consistent with this previous research, though it is notable that we observed decreased *Bdnf* expression in hippocampal tissue of B6 MS-reared females whereas increased *Bdnf* DNA methylation was only observed in MS-reared Balb/c mice. Intriguingly, we found increased *Bdnf* expression in the prefrontal cortex of MS-reared Balb/c mice that was not associated with changes in DNA methylation of the examined CpG sites. The lack of correspondence between expression and DNA methylation highlights the complex regulatory networks that may be recruited by MS-rearing and may vary over time. For instance, although DNA methylation changes have the potential to induce long-lasting changes in gene expression (50), it is possible that compensatory mechanisms may override the effect of DNA methylation on gene regulation. In addition, the behavioral testing of these individuals, which may alter gene expression and DNA methylation independent of rearing condition, may have limited our ability to provide a clear correlation between MS-induced DNA methylation and gene expression. However, it is important to acknowledge that DNA methylation is only one of many epigenetic mechanisms that can regulate gene expression and so it may be the case that variation in DNA methylation is not causally related to the gene expression changes we observed in the current study.

It is also worth noting the limitations of our gene expression/epigenetic analyses. First, we examined only total *Bdnf* mRNA levels and it is possible that changes in specific (particularly low-abundance) *Bdnf* transcripts were not detected due to a dilution effect. In addition, we examined only DNA methylation of the CpG sites in the *Bdnf* promoter regions IV and IX, previously shown to be epigenetically regulated (51, 69, 70). Thus, it is possible that MS could have induced epigenetic changes in *Bdnf* promoter regions not examined in this study. There is increasing evidence for epigenetic variation at CpG shores rather than promoter CpG islands (80) and so loci outside of the regions analyzed might be more relevant to MS-induced effects. Finally, the DNA methylation changes we observed were modest and it is difficult to evaluate the biological relevance of changes of this magnitude derived from the current methodological approaches used for *in vivo* analyses. It seems likely that MS-induced epigenetic effects are specific to a subpopulation of cells within the brain regions examined and thus are diluted through the inclusion of multiple neuronal and glial cells. Therefore, future studies of MS-induced epigenetic changes would benefit from cell-type specific analyses that may facilitate our efforts to detect epigenetic and gene expression changes that are induced by early life adversity and contribute to behavioral abnormalities occurring later in life.

The rapid development of methodologies for assessing epigenetic variation has also provided opportunities to determine the translational relevance of research on adversity-induced changes in DNA methylation. In post-mortem brain tissue, increased hippocampal DNA methylation of the *Nr3c1* promoter and decreased *Nr3c1* expression is observed in individuals with a history of childhood abuse (23). Similar adversity-associated increases in *Nr3c1* promoter methylation have been documented in humans in non-neuronal tissues such as fetal cord blood (81, 82), blood lymphocytes (83), and buccal cells (84). Genome-wide DNA methylation analyses of blood lymphocytes suggest that global DNA hypermethylation may result from childhood social/maternal deprivation (being reared in an institution vs. reared by biological parents) (24). The question raised by these intriguing findings is the relevance of peripheral epigenetic markers for predicting epigenetic variation in the brain – particularly in light of the goal to further our understanding of the neurobiological pathways through which adversity leads to psychopathology. We have previously found limited concordance between peripheral and brain tissues in DNA methylation levels of the *Nr3c1* gene promoter (67). Within the current study, though peripheral tissues were not assessed, it is clear that MS has a unique epigenetic impact in different brain regions (i.e., *Nr3c1* and *Bdnf* MS-associated DNA methylation changes observed in the hippocampus and not the prefrontal cortex). Thus, even within the brain, epigenetic responsiveness may not be consistent across genes. This observation does not invalidate approaches using peripheral tissue to predict neuronal changes but does suggest that the complexity of tissue-specific molecular responses and the mechanisms through which both peripheral and brain tissues would be affected by adverse environmental experiences need to be carefully considered.

SEX-SPECIFIC OUTCOMES ASSOCIATED WITH ADVERSITY

Sex differences in response to early life experiences are a relatively consistent finding within the literature. In humans, childhood maltreatment may increase rates of depression and drug use in females, with more limited effects in males (85). On neuroendocrine measures, sex is a significant modulator of the relationship between childhood adversity and HPA activity (86). This sex-specificity is also observed following prenatal adversity. Exposure to *in utero* stress/nutrient deficiency during pregnancy may increase the risk of schizophrenia in males but not females (87) and maternal bereavement stress during pregnancy has been found to increase the risk of attention deficit disorder in males (88). However, these effects may be due in part to the sex-bias in these disorders induced by hormonal and genetic differences (with males having higher rates than females) (89). Animal studies likewise suggest the sex-specificity of early life adversity (42) and in the current study, sex by rearing condition interactions are the norm rather than the exception. Similar to the effect of strain, our findings support the hypothesis that both males and females are sensitive to the effects of MS, but that the effects of MS manifest in different ways dependent on sex. We have found that B6 males, but not B6 females, exhibit hyperactivity in response to MS. Similarly, Balb/C males are more vulnerable to MS-induced anhedonia than Balb/C females. In addition, we have recently shown that the differential response of males and females to early life toxicological exposures can be observed at the level of gene expression and DNA methylation in the brain corresponding to changes in social and anxiety-like behavior (44). Sex-specific epigenetic effects are an emerging theme in the study of early life adversity and may account for the sex-bias in adversity-associated behavioral and neurobiological dysfunction. Interestingly, in the current study we observed sex-specific gene expression and epigenetic variation in B6 mice, whereas in Balb/c mice, male and female effects of MS-rearing are similar. Consistent with previous reports (54), we also find sex differences in DNA methylation regardless of rearing condition. These findings add another layer of complexity, which

includes differential genetic background, to the investigation of sex-specific responsiveness to adversity.

CAN ADVERSITY LEAD TO IMPROVED OUTCOMES?

Though the experience of disruption to the *in utero* environment or childhood maltreatment is linked to psychiatric dysfunction (7, 8), it is clear that there is a significant degree of resilience to early life adversity (15, 90). Within the current study, the effects of MS-rearing are relatively modest suggesting that, similar to human populations, many individuals are resilient to MS. However, in addition to these indices of resilience, we find that MS-reared Balb/c females will more rapidly enter the anxiogenic center area of the open-field and have increased sucrose preference. These behavioral phenotypes would suggest reduced anxiety- and depressive-like phenotypes as a function of early life adversity. In light of these perplexing findings, one hypothesis is that adversity can lead to improved outcomes dependent on sex and genetic background. Several lines of evidence may be relevant to evaluating the plausibility of this hypothesis. In primates, early, intermittent periods of MS have been documented to reduce indices of anxiety-like behavior and enhance HPA negative-feedback, suggesting a protective effect of early life adversity (91–93). There is also evidence for enhancements in functioning following exposure to adverse experiences, if the adversity is constant across developmental periods. Though maternal depression during pregnancy can predict impairments in functioning, there is enhanced motor and neuronal development in infants that experienced maternal depression during both *in utero* and postnatal periods (compared to infants who were only exposed to maternal depression at one developmental timepoint) (94). Previous studies of Balb/c mice have shown that the *in utero* environment of this mouse strain can exert significant programing effects, leading to increased anxiety-like behavior (95). It may be the case that MS during postnatal development in this strain generates a better environmental "match" to the prenatal environment, allowing the neuroendocrine adaptations of offspring to enhance functioning. Though these are hypotheses that have yet to be tested, the phenomenon of improved functioning following adversity in a subset of individuals should not be dismissed.

INTER-INDIVIDUAL VARIABILITY IN THE EFFECTS OF MATERNAL SEPARATION

The relatively modest effects of MS-rearing that we observe in the current study and the inconsistent effects of MS observed in previous studies (17) requires careful examination of the MS paradigm and the hypothesized pathways through which this form of adversity alters offspring development. Prolonged separations between mothers and offspring are thought to model childhood neglect and the stress of this manipulation has been found to reduce mother-infant interactions during the post-reunion period (96). However, these group-level effects may not be observed in all litters and certainly there are individual differences in the frequency of mother-infant interactions under standard rearing conditions that have significant programing effects on brain and behavior (97, 98). These individual differences in maternal behavior likely contribute to the variability in response to adversity. The use of MS combined with maternal stress during the separation period is one approach intended to create a more consistent reduction in maternal behavior in MS litters and this methodology has previously been found to reduce mother-infant interactions in mice (21). However, this approach does not account for the variability in maternal care in control litters and does make the interpretation of the role of MS vs. maternal care on outcome measures problematic. This will be an important issue to address in subsequent studies using the current MS protocol.

A second issue to consider within the MS paradigm is how the individual responsiveness to adversity may be used to better understand the molecular and neurobiological basis of risk and resilience. In the current study, we examined gene expression and DNA methylation in a random subset of individuals. However, perhaps a more powerful strategy for assessing the link between adversity, neurobiological changes, and risk phenotypes would be to stratify the sample with comparisons between those individuals that manifest risk phenotypes (increased anxiety- and depressive-like behavior) and those individuals that are resilient. Within the context of studies aimed at understanding the etiological pathways leading to psychopathology, this approach, combined with a more detailed assessment of the characteristics of the postnatal environment, may provide a more informative experimental paradigm that can advance our understanding of the biological basis of adversity-induced dysfunction.

FUTURE DIRECTIONS

The strain and sex-dependent effects of MS that we identified in the current study highlight the complexity of the effects of early life adversity. Though strain and sex differences in neurobiology and behavior are well documented, the molecular basis of the differential response to environmental exposures has yet to be elucidated. Epigenetic analyses within future studies of these effects may advance our understanding of this differential response and should be combined with experimental designs where important modulating variables, such as prenatal and postnatal maternal effects, are assessed. Within-individuals, the differential epigenetic response of different tissues (brain and peripheral) over multiple timepoints may provide important insights into the pathways leading to risk phenotypes and contribute to translational studies of the impact of early life adversity.

MATERIALS AND METHODS

ANIMALS

C57BL/6J (B6) and Balb/cJ (Balb/c) mice (Jackson Laboratories) were used in these studies. Adult males ($n = 10$) and females ($n = 20$) of each strain were housed two per cage in $10.5'' \times 19'' \times 6''$ cages and habituated to the animal facility in the Department of Psychology at Columbia University for 2 weeks prior to mating. At mating, two females were housed with one male for 10 days. This mating protocol generated $n = 13$ B6 and $n = 14$ Balb/c litters. At birth (PND0), all pups were counted and weighed. Animals were maintained at a constant temperature and humidity with a 12L:12D light schedule (lights off 10:00 a.m.) and *ad libitum* access to chow and water. All procedures were performed in accordance with guidelines of the NIH regarding the Guide for the Care and Use of Laboratory Animals and with the approval

of the Institutional Animal Care and Use Committee (IACUC) at Columbia University.

POSTNATAL MATERNAL SEPARATION

Starting on PND1, litters were exposed to daily MS or standard laboratory rearing conditions (see **Figure 1**). The protocol, previously used in (21), involved 2 h of daily separation of pups and dam from PND1 to PND14 combined with maternal exposure to unpredictable stress during the period of separation. At the start of the separation period, dams were removed from the home-cage and placed in a clean cage with *ad libitum* access to chow and water. Pups were also removed from the home-cage and placed together in a clean cage. At a randomly selected time within the 2-h separation, dams were exposed to 20 min of restraint stress or 2 min of forced swim. During restraint, females were removed from the temporary housing cage and placed in a conical tube that restricted all vertical and horizontal movement. During forced swim, mice were placed in a 2 l glass beaker containing 1 l of water (20°C). After the 2-min period, mice were patted dry with a towel and returned to the temporary housing cage.

REPRODUCTIVE OUTCOMES

The breeding protocol used in the current study resulted in a 65 and 70% rate of successful births in Balb/c and B6 mice, respectively. Average litter weights at PND0 and PND6, litter size at PN6, litter mortality rates during the first postnatal week, litter sex ratio, and average weaning weights of male and female offspring are provided in **Table 3**. No significant rearing condition effects were observed except on the measure of male pup weaning weights, which were decreased in MS-reared Balb/c males compared to control-reared Balb/c males [$t(1, 12) = 3.03$, $p < 0.05$]. Litters containing fewer than two pups at the time of weaning (PND28) were excluded, resulting in $n = 6$ litters per strain for the control rearing condition and $n = 8$ B6 and $n = 7$ Balb/c litters for the MS-rearing condition. For behavioral measures, one to two pups per sex per litter were tested (*B6*: control male, $n = 10$; control female, $n = 9$; MS male, $n = 7$; MS female, $n = 11$; *Balb/c*: $n = 10$/sex/rearing condition). For these analyses, litter was used as a covariate. For gene expression and DNA methylation analyses, only one pup (per sex) was used per litter with a sample size of $n = 6$ pups per sex per rearing condition.

Behavioral assessment

At PND28, all offspring were weaned and commenced behavioral testing at PND35 (see **Figure 1**). All offspring underwent testing in the open-field apparatus (PND35), assessed for sucrose preference (PND36–39), and then observed during a dyadic social encounter with a stimulus mouse in the open-field apparatus (PND40). Testing during juvenile/adolescent development was conducted to determine the emergence of behavioral risk phenotypes at this early period, prior to the onset of full sexual maturity, and create further parallels with studies in humans that have observed childhood and adolescent behavioral problems that are predicted by adversity and predictors of later-life risk of psychopathology (99–101). However, it should be noted these behavioral tests have been validated in adult rather than juvenile/adolescent mice.

OPEN-FIELD TESTING

The open-field apparatus used was a $24'' \times 24'' \times 16''$ black plastic box. On the day of testing, the mouse was placed directly into one corner of the open-field. After a 10-min session, the mouse was returned to its home-cage. All testing was conducted under red lighting conditions and tests were video recorded. Behaviors scored using Ethovision (Noldus) included: (1) distance traveled, (2) latency to enter the center area, and (3) center area exploration (time spent in the inner $12'' \times 12''$ area).

SUCROSE PREFERENCE

Immediately following open-field testing, mice were singly housed and placed in a cage with two water bottles (both containing water). The following day, on PND36, both bottles were removed. One bottle was filled with water, weighed, and placed in the cage. The second bottle was filled with a 1% sucrose solution, weighed, and placed in the cage. Each day, bottles were weighed to determine consumption levels (three consecutive days). The position of the sucrose vs. water bottle was alternated each day to avoid place preference. Sucrose preference was defined as having average sucrose consumption levels (averaged across the 3-day period) of 75% or higher. Percentage consumption levels were defined as total sucrose consumed divided by the total volume of liquid consumed (water + sucrose). Sucrose preference was stable over consecutive days in both control and MS mice suggesting that initial reactivity to single housing (conducted on the day prior to sucrose preference testing) did not contribute to the rearing condition effects observed.

SOCIAL BEHAVIOR

At PND40, a subject mouse was placed in the open-field apparatus with a same-sex stimulus mouse (129Sv) for 30 min. Sessions were video recorded. Latency to sniff/approach the stimulus and

Table 3 | Reproductive outcomes (mean ± SEM) in control and MS litters.

		Av. birth weight	PN6 litter size	PN6 pup av. weight	Litter sex ratio (m/f)	% Pup mortality[1]	Av. weaning weight (m)	Av. weaning weight (f)
B6	Control	1.27 ± 0.03	6.00 ± 0.82	3.04 ± 0.40	1.11 ± 0.70	13.65 ± 4.67	15.85 ± 1.08	13.47 ± 0.27
	MS	1.32 ± 0.05	5.14 ± 0.83	3.61 ± 0.25	0.90 ± 0.86	22.02 ± 8.94	15.55 ± 0.61	17.85 ± 4.38
Balb/c	Control	1.36 ± 0.06	6.00 ± 0.63	3.55 ± 0.50	1.22 ± 0.65	13.16 ± 6.41	**15.40 ± 0.44**	14.20 ± 0.92
	MS	1.41 ± 0.04	5.75 ± 0.65	3.68 ± 0.30	1.01 ± 0.89	5.90 ± 3.87	**13.36 ± 0.50***	13.16 ± 0.50

*Statistically significant MS-induced effects are indicated in bold font; *p < 0.05 control vs. MS; [1]mortality occurring between PN0 and PN6 (no mortality was observed after this period).*

occurrence of aggressive behaviors (tail rattling, chasing, biting) were coded.

NUCLEIC ACID ISOLATION

Following assessment of social behavior at PND40, mice were sacrificed by rapid decapitation and brains extracted and stored at −80°C. Whole hippocampus and cortical tissue containing the prefrontal cortex were dissected from partially thawed tissue and Allprep DNA/RNA mini kit (Qiagen) was used for simultaneous extraction of total RNA and genomic DNA.

QUANTITATIVE REAL-TIME PCR

Gene expression was assessed using reverse transcription (The SuperScript® III First-Strand Synthesis System, Invitrogen) followed by quantitative real-time PCR with a 7500 real-time PCR system (Applied Biosystems). Using specific primer sets (see **Table 4**), mRNA levels of the glucocorticoid receptor (*Nr3c1*) and brain-derived neurotrophic factor (*Bdnf*) were determined. Relative mRNA expression was calculated using the standard ΔΔCT method (102) with male control samples as a reference sample and cyclophilin A (*CypA*) and beta-actin (*Actb*) as endogenous reference genes.

BISULFITE-PYROSEQUENCING

DNA methylation at specific CpG sites in the *Nr3c1* and *Bdnf* genes was analyzed using bisulfite-pyrosequencing method. Bisulfite conversion of DNA samples (500 ng) was carried out using EpiTect Bisulfite Kit (Qiagen). Biotinylated PCR products were obtained using PyroMark PCR kit (Qiagen) and PCR primers specific for *Nr3c1* and *Bdnf* gene regions (see **Figure 4**). Pyrosequencing was performed on a PyroMark Q24 Pyrosequencer using specific pyrosequencing primers (see **Table 5**). Average DNA methylation levels of CpG sites were quantified using PyroMark Q24 2.0.4. Software (Qiagen).

STATISTICAL ANALYSES

Consistent with previous studies examining strain differences in behavior, in our preliminary analyses we found significant effects of strain in all behavioral tests conducted, with B6 mice exhibiting increased time spent in the center area of the open-field ($p < 0.001$), longer latencies to enter the inner area ($p < 0.001$), increased average sucrose consumption ($p < 0.05$), and a decreased likelihood of engaging in aggressive behavior ($p < 0.05$) compared to Balb/c mice. Thus, for analyses of rearing condition effects, we analyzed each strain separately. Open-field data (time spent in the center area, total activity) were analyzed using 2-way ANOVA, with sex and rearing condition as independent variables and litter as a covariate. Latency data (time to enter the center area, social approach) were analyzed with Kaplan–Meier survival analysis. For sucrose consumption data, a χ^2 test was conducted to determine group differences in likelihood of exhibiting sucrose preference (>75% sucrose consumption). Similarly, a χ^2 test was conducted to determine group differences in likelihood of engaging in aggressive behavior (males only). For gene expression and DNA methylation analyses, we found significant strain by brain interactions and analyzed data from each strain and brain region using separate 2-way ANOVAs with sex and rearing condition as independent variables. For DNA methylation analyses, average CpG methylation levels across the multiple CpG sites assessed was used in the ANOVA.

Table 4 | Primers for gene expression analyses.

Gene name	Forward primer	Reverse primer
Nr3c1	AACTGGAATAGGTGCCAAGG	GAGGAGAACTCACATCTGGT
Bdnf	CATAAGGACGCGGACTTGTACA	AGACATGTTTGCGGCATCCA
CypA	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
Actb	TATTGGCAACGAGCGGTTCC	TGGCATAGAGGTCTTTACGG ATGTC

Table 5 | PCR and pyrosequencing primers used for DNA methylation analysis.

MOUSE GR GENE (*Nr3c1*) – chr18:39,649,906-39,650,025*	
PCR primer – forward	GGTTTTGTAGGTTGGTTGTTATTT
PCR primer – reverse – Biotinylated	/5Biosg/TCTCTTCTCCCTAACTCCTT
Pyrosequencing primer	GGGTTTTGGAGGTAGATTTA
MOUSE BDNF PROMOTER IV (*Bdnf IV*) – SITES IV1-IV4 – chr2:109,532,399-109,532,715*	
PCR primer – forward	TAGGATTGGAAGTGAAAATATTTATAAAGT
PCR primer – reverse – Biotinylated	/5Biosg/CCTTCAACCAAAAACTCCATTTAATCT
Pyrosequencing primer	AGAGGAGGTATTATATGATAG
MOUSE BDNF PROMOTER IX (*Bdnf IX*) – SITES IX5-IX1 – chr2:109,562,918-109,563,064*	
PCR primer – forward	GGTGTTTGGTGTTTTAAGTAGTT
PCR primer – reverse – Biotinylated	/5Biosg/ACAAATCCTATATAACCTTTTAATTCC
Pyrosequencing primer	TGAGTAGGAGTAGTATGATAA

**Genomic coordinates are based on the UCSC Genome Browser Mouse July 2007 (NCBI37/mm9) Assembly.*

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From resilience to vulnerability: mechanistic insights into the effects of stress on transitions in critical period plasticity

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While early experiences are proposed to be important for the emergence of anxiety and other mental health problems, there is little empirical research examining the impact of such experiences on the development of emotional learning. Of the research that has been performed in this area, however, a complex picture has emerged in which the maturation of emotion circuits is influenced by the early experiences of the animal. For example, under typical laboratory rearing conditions infant rats rapidly forget learned fear associations (*infantile amnesia*) and express a form of extinction learning which is relapse-resistant (i.e., extinction in infant rats may be due to fear erasure). In contrast, adult rats exhibit very long-lasting memories of past learned fear associations, and express a form of extinction learning that is relapse-prone (i.e., the fear returns in a number of situations). However, when rats are reared under stressful conditions then they exhibit adult-like fear retention and extinction behaviors at an earlier stage of development (i.e., good retention of learned fear and relapse-prone extinction learning). In other words, under typical rearing conditions infant rats appear to be protected from exhibiting anxiety whereas after adverse rearing fear learning appears to make those infants more vulnerable to the later development of anxiety. While the effects of different experiences on infant rats' fear retention and extinction are becoming better documented, the mechanisms which mediate the early transition seen following stress remain unclear. Here we suggest that rearing stress may lead to an early maturation of the molecular and cellular signals shown to be involved in the closure of critical period plasticity in sensory modalities (e.g., maturation of GABAergic neurons, development of perineuronal nets), and speculate that these signals could be manipulated in adulthood to reopen infant forms of emotional learning (i.e., those that favor resilience).

Keywords: maternal-separation, FGF2, fear conditioning, memory retention, extinction, development, infant, critical period

INTRODUCTION

Early life experiences have long been considered critical for the establishment of mental health. Exposure to a range of childhood adversities such as maladaptive family functioning, rearing in an institutional setting, and trauma lead to increased mental health risk and difficulties in emotional regulation and cognitive functioning (1–5). In both humans and non-human species the early rearing environment has been shown to influence the development of brain regions critical to emotional processing and/or mental health outcomes (3, 6–8). Despite the recognized importance of early life experiences in the establishment of mental health however, there has been surprisingly little empirical research which examines the role of early experiences (such as adverse rearing) on the development of emotional learning. Yet some forms of emotional learning (e.g., learning to fear and learning to inhibit fear responses) are critically involved in both the establishment and treatment of mental health disorders in humans [see (9), for a review (10–12)]. Further, evidence from animal models has demonstrated considerable developmental heterogeneity in the processes involved in fear learning and fear inhibition (13–18). Hence, understanding the maturation of emotional learning and how its developmental trajectory is altered by different early experiences might aide in our understanding and treatment of mental health disorders across the lifespan.

In this review we describe the normal trajectory of fear learning across the infancy to juvenile periods of development in the rodent and discuss how developmental dissociations in these learning processes are altered by a variety of early life experiences (specifically, exposure to early life adversity or fibroblast growth factor-2; FGF2). Considering the high degree of similarity in fear learning outcomes following early manipulation of the rearing environment and FGF2, we propose a model via which the experience of early adversity might activate, within the limbic circuit, molecular signals known to be involved in critical periods of plasticity in other brain regions via an FGF2-dependent pathway. The review ends with a discussion on how the proposed model might guide further pre-clinical research in this field as well as highlighting potential areas for translation to humans.

DEVELOPMENTAL DIFFERENCES IN FEAR LEARNING

In recent years, studies using Pavlovian fear conditioning have demonstrated a number of fundamental differences in emotional learning in infant and adult animals. During a typical Pavlovian fear conditioning procedure an initially neutral conditioned stimulus (CS; e.g., noise) is paired with an aversive unconditioned stimulus (US; e.g., footshock). Such pairings rapidly lead the animal to express a species-specific defensive/fear response toward the CS [e.g., freezing in the rat; (19)]. Although both infant and adult rodents can learn a CS-US association during fear conditioning, their retention of those fear memories differs dramatically. Specifically, following fear conditioning adult rats will typically express fear to that cue for the rest of their life (20). Infant rats, on the other hand, exhibit rapid forgetting, a phenomenon known as *infantile amnesia* (13). For example, when given two pairings of a white noise CS with a foot shock US, both infant [i.e., postnatal day (P) 16] rats and juvenile (i.e., P23) rats show equivalent levels of fear immediately after training (18). However, when tested 2 days after training, infants show a dramatic decrease in fear, while juveniles continue to express a high level of fear in the presence of the CS. This suggests that while infant animals can acquire fear just as readily as older animals, they do not retain the memory across an extended period of time (13, 21, 22). This profound and spontaneous forgetting is not limited to infant rats but is experienced by all altricial animals, including humans (23). For example, humans are generally unable to recall events that occurred prior to the age of 3 years and have hazy memories of events that occurred until around 5–6 years of age (24).

One question of interest to neuroscience researchers is what happens to the memory trace following infantile amnesia. That is, does the forgetting represent decay in the memory trace, leading to eventual erasure of that memory, or are infant memories simply unable to be retrieved? The evidence suggests that infantile forgetting often represents a retrieval failure. Numerous studies have shown that a pre-test reminder treatment effectively reverses the deficit in retention, suggesting that infantile amnesia is caused by a failure of cues to spontaneously retrieve the memory trace (15, 25–27). In addition, reducing GABAergic inhibition in the infant rat at test (via systemic injection of FG7142; a partial inverse agonist of the $GABA_A$ receptor) leads to a forgotten memory being expressed (15, 28). Interestingly, studies have shown that administration of midazolam, which increases $GABA_A$ activity, in adult rats has strong amnestic effects (29), suggesting that infantile forgetting may be an exaggerated form of adult memory loss.

DEVELOPMENTAL DIFFERENCES IN FEAR INHIBITION

Another area where developmental differences are observed is in the inhibition of fear. That is, once fear is acquired it can then be decreased or inhibited through a process known as extinction. During a typical extinction procedure the animal is repeatedly exposed to the CS without the reinforcing US (e.g., shock). In the last decade, extensive research has been conducted examining the behavioral, neural, and molecular mechanisms underlying fear extinction. On a behavioral level, it is widely accepted that extinction in older animals (e.g., juvenile and adult animals) is not simply erasure of the original fear memory. Instead, extinction is believed to involve the formation of a new inhibitory (CS-noUS) memory. Evidence for the "new inhibitory learning" account of extinction comes from both rodent and human studies showing that fear can return following extinction training through either a change in context [renewal; e.g., (30, 31)], presentation of an aversive stimulus [reinstatement; e.g., (32, 33)], or simply the passage of time [spontaneous recovery; e.g., (33, 34)]. Thus, in older animals, extinction is relapse-prone.

The idea that extinction involves new learning in juvenile and adult animals is further supported by evidence from pharmacological studies demonstrating that extinction involves the same cellular mechanisms as other forms of new learning. For instance, both fear conditioning and fear extinction require activation of the *N*-methyl-D-aspartate receptor (NMDAr), as administration of DL-2-amino-5-phosphonovaleric acid (APV; an NMDAr antagonist) either systemically or directly into the brain disrupts both forms of learning (35–37). Conversely, systemic or intra-amygdala administration of the NMDAr partial agonist D-cycloserine (DCS) enhances extinction retention (38–40). Other cellular mechanisms involved in the mature form of fear extinction have also been explored. For instance, along with NMDAr transmission, fear extinction in juvenile/adult rats has been shown to rely on GABAergic (41, 42) and opioidergic transmission (43, 44).

The characteristics of extinction in infant rodents have also begun to be explored and the results suggest that infant rodents exhibit a qualitatively different extinction profile compared to juvenile and adults. Whereas adult animals exhibit relapse-prone extinction, infants exhibit *relapse-resistant* fear extinction. That is, infant P17 animals do not show renewal, reinstatement, or spontaneous recovery following extinction (16, 42, 45, 46). The lack of relapse behavior seen after extinction in the young animal suggests that extinction at this age is mediated by a fundamentally different mechanism, which might be best characterized as erasure of the original fear memory rather than new learning. In support of this possibility, other studies have demonstrated that extinction in infant animals is not dependent on NMDArs (47); in contrast to P24 rats, systemic administration of the NMDAr antagonist MK-801 did not impair extinction retention in P17 animals. This effect is not due to a generalized lack of NMDAr-involvement in infant learning because the same drug was shown to impair fear acquisition in rats when given prior to conditioning in infancy. These findings suggest that while NMDArs are involved in some forms of learning during infancy (i.e., fear conditioning), they are not involved in others (i.e., fear extinction).

Other neurotransmitters have also been shown to differentially modulate early extinction memories. For instance, unlike juvenile and adult rats, GABAergic transmission does not affect long-term extinction in infant rats (42), suggesting that extinction does not involve formation of a new "inhibitory" association in young rats. On the other hand, some neurotransmitter systems do appear to be involved in extinction across age. Specifically, endogenous opioids appear to regulate extinction in infant animals, as P17 rats given the opioid receptor antagonist naloxone exhibited impaired within-session extinction compared to animals given saline (44); a finding which is similar to that seen in adult rats (43).

The developmental differences in fear inhibition are not only observed on the behavioral and pharmacological levels as there are also marked differences in the neural circuitry which supports

extinction across development. In adult animals, lesion, immunohistochemical, and electrophysiological studies have implicated the amygdala, medial prefrontal cortex (mPFC), and hippocampus in the extinction of fear [see (48–50), for extensive reviews on the role of these structures in extinction]. Specifically, a widely accepted neural model of extinction proposes that the amygdala is involved in the acquisition and consolidation of learned fear [e.g., (51)], while the mPFC is important for regulating the expression of fear through either inhibiting or exciting amygdalar neuron output [e.g., (50)]. Additionally, the hippocampus appears to be involved in the contextual modulation of extinction through its projections to the mPFC (52, 53).

While this neural model of extinction has been predominantly based on rodent studies, there is evidence to suggest that a similar circuitry is involved in regulating emotional memories in humans (54). For example, Phelps and colleagues showed that the mPFC-amygdala circuit is activated in humans following extinction training (55), while Kalisch et al. (56) found that retrieval of a context-dependent extinction memory activated the hippocampal-mPFC circuit. Interestingly, this "extinction circuit" has been shown to be dysfunctional in individuals with post-traumatic stress disorder (PTSD). Specifically, some studies have found that individuals with PTSD exhibit *hypoactivation* of the fear inhibition components of the circuit (i.e., mPFC and hippocampus) and *hyperactivation* of the fear activation components of the circuit (i.e., amygdala), relative to healthy controls [e.g., (12, 57)].

While the extinction circuit has been well documented in adult rodents and adult humans, until very recently this circuit had not been examined at earlier stages of development. Over the past 5 years, however, some progress has been made in mapping the neural circuitry mediating extinction in the developing animal. Those studies indicate that if extinction occurs in the juvenile stage of development, then it involves the same neural circuit as extinction in adulthood. In contrast, extinction in the infant stage of development appears to involve a different circuit. For example, Kim and Richardson (58) found that inactivating the amygdala (via infusion of the $GABA_A$ agonist muscimol) prior to extinction significantly impaired long-term extinction in both P24 and P17 rats. Further, it was observed that there was an increase in the number of phosphorylated mitogen-activated protein kinase (pMAPK) neurons in the basolateral amygdala (BLA) following extinction training in rats of both ages (59). Therefore, it seems that the amygdala is an important structure for the extinction of conditioned fear in rats, regardless of age. In contrast, the mPFC appears to mediate fear extinction only in older animals [i.e., juveniles and adults; (59)]. In that study, infusion of muscimol into the mPFC prior to extinction training impaired extinction retention in P24 rats but not in P17 rats. In addition, while extinction training caused an increase in pMAPK-labeled neurons in the mPFC of P24 rats, there was no extinction-related change in pMAPK-labeled neurons in that structure in younger animals. Together, the research on fear extinction in the infant rat appears to suggest that infants recruit a much simpler neural circuit during extinction than do rats extinguished at later stages of development (i.e., juvenile through to adulthood). It has been proposed that these neural differences in extinction might underlie the less flexible extinction behavior seen in infant rats. That is, perhaps the lack of relapse following infant extinction in the rat is the outcome of a simple extinction circuit which cannot integrate multiple, contextually gated associations. See **Table 1** for a summary of the behavioral and neural differences in extinction and fear retention across development.

Table 1 | Summary of the behavioral and neural characteristics of the fear retention and extinction systems in adult and infant (<P21) rodents.

	Adult rodent	Infant rodent	Infant rodents following early stress/CORT/FGF2
Renewal	✓	×	✓
Reinstatement	✓	×	✓
NMDA	✓	×	?
GABA	✓	×	✓
Endogenous opioids	✓	✓	?
Amygdala	✓	✓	?
mPFC	✓	×	?
Good fear retention	✓	×	✓

✓ Indicates that the phenomenon is present in age or treatment groups; × indicates that the phenomenon is absent in age or treatment groups; ? indicates that the phenomenon has not yet been examined in the age or treatment group. See text for definition of the terms used in this table.

The current literature clearly indicates that fear retention and fear inhibition are dynamic processes that exhibit considerable developmental heterogeneity. Whereas infant rats exhibit marked forgetting and use a simpler extinction system characterized by a resistance to relapse, older rats demonstrate better memory retention and use a more flexible neural circuit that results in relapse-prone extinction learning. While examination of these differences has occurred primarily in animal models, there is evidence that at least one of the transitions (i.e., the transition from infantile amnesia to adult-like memory retention) also occurs in developing humans. It is now commonly accepted that memories formed before the age of approximately 3 years in humans are generally inaccessible to conscious recollection in adulthood [e.g., (24, 60)]. While much of the human research on infantile amnesia has focused on various cognitive factors that might contribute to the effect [e.g., language acquisition, development of self-concept, increasing ability to utilize reminder cues; (61–63)], the occurrence of the same effect in non-human animals suggests that more basic neurobiological mechanisms might provide a better account for infantile amnesia. In contrast to the complimentary findings on infantile amnesia across rodents and humans, there hasn't been any, to our knowledge, research examining whether the transition from relapse-resistant to relapse-prone extinction is also a feature of human development. Future studies should examine whether the transition in extinction mechanisms also occurs in humans.

The fact that developmental transitions in emotional learning take place in humans as well as rodents is of particular interest, suggesting that findings in either species might be successfully translated to the other. Indeed, a mechanistic understanding of the developmental transitions in emotional learning across species might have considerable clinical implications because anxiety disorders are characterized by persistent expression of fear and

high rates of treatment relapse. In an effort to uncover some of the mechanisms which regulate the expression of infant fear learning within a rodent model, some very recent studies have begun to examine factors which are involved in the transition from infant- to adult-like fear learning, with a view to manipulating these mechanisms in adulthood to promote infant-like forgetting and relapse-resistant extinction.

EARLY EXPERIENCES REGULATE THE TRANSITION BETWEEN INFANT- AND ADULT-LIKE FEAR LEARNING IN RODENT MODELS

Two different types of early experience have recently been shown to affect the age at which rats transition between infant- and adult-like fear learning. While these experiences are vastly different in nature, they both appear to impact the developmental transition in fear learning in similar ways (i.e., both manipulations lead to early expression of adult-like fear retention and extinction behaviors).

STRESS

It has been known for decades that exposure to stressors or stress hormones (corticosterone; CORT) can program the maturation of fear responding. For example, rats begin to exhibit species-specific defense responses (freezing, inhibition of ultrasonic vocalizations; USV) to the presence of a strange adult male/male odor at approximately P10. Further, while the amygdala is not activated by the presentation of a male odor in rats younger than P10, amygdala activation is increased following presentation of the same stimulus in rats aged P10 and older (64–66). Defense responding and amygdala activation can be elicited by presentation of a potential predator odor earlier if rats are given exogenous CORT at P8. Further, these responses can be delayed if rats are adrenalectomized, which leads to a reduction in CORT [i.e., removal of the adrenal gland and subsequent reduction in CORT; (66–69)].

In addition to the stress-induced acceleration of unlearned fear reaction development, the maturation of learned fear reactions also appears to be affected by stress exposure. For example, in the second postnatal week of life rats exhibit a developmental transition in their behavioral and neural response to an odor previously paired with shock. Specifically, in rats aged P10 and older odor-shock conditioning leads to subsequent avoidance of the shock-paired odor and activation of the amygdala. However, rats conditioned at P6–P8 exhibit a paradoxical approach response toward the odor (70, 71). In addition, presentation of the shock-paired odor does not lead to increased activity in the amygdala of P8 rats (72), suggesting that different neural structures are involved in the conditioned responses exhibited by P10 and P8 rats. Interestingly, if rats were raised in a stressful rearing environment, or were given a CORT injection before test, then a precocious avoidance response to the shock-paired odor was observed at P8, which was correlated with increased amygdala activity (72–75). Thus, early life stress in rodents accelerates the transition between infant- and adult-like behaviors and neural responses in odor-shock associative learning just like it accelerates the development of unlearned fear responses to a potential predator odor.

Although environmental effects on the maturation of fear responses have been investigated for some time, how the environment affects development of fear retention and fear extinction has only recently begun to be investigated. Interestingly, those studies show that early exposure to stress or CORT also accelerates the maturation of fear retention and extinction learning. Specifically, compared to a group of standard-reared (SR) infant rats, infants exposed to maternal-separation (MS; 180 min separation from P2 to P14) before conditioning on P17 express fear memories for longer periods of time (76). While SR infants forgot a conditioned association in as little as 10 days, MS infants expressed memory for the conditioned association up to 30 days after training. Similarly, pups that were suckled by a SR mother that had been exposed to CORT in her drinking water (from P2 to P14), but not pups suckled by vehicle-exposed mothers, also exhibited longer retention of fear memories. Taken together, those results suggest that early stress/CORT exposure leads to an accelerated transition in the fear retention system used by infant rats. In other words, rats make a precocious transition from the infantile amnesia system to the adult-like retention system following exposure to stress/CORT.

It is not only an early transition into adult-like retention that is seen following MS however. In another set of studies the effect of MS on the expression of two relapse phenomena after extinction (fear renewal and reinstatement) was examined in infant rats (77). It was shown that while the SR infant rats did not exhibit either of those relapse phenomena [replicating past findings in P17 rats; (16, 45)], the MS infants did. In other words, following MS rats made an early transition from the infant relapse-resistant extinction system to the adult-like relapse-prone extinction system. In addition to exhibiting increased relapse, the expression of extinction in MS P17 rats was also found to be dependent on activation of $GABA_A$ receptors. As mentioned earlier, the expression of adult extinction memories requires activation of the $GABA_A$ receptors (41). Similar to studies in adults, when GABAergic inhibition was decreased at an extinction test in juvenile rats (via injection of FG7142), extinction retention was impaired (42). However, in that study FG7142 had no effect on levels of expressed fear in infant rats. That is, infant rats exhibited low levels of freezing at test following extinction regardless of whether they received FG7142 or not. Interestingly, when MS infant rats were given FG7142 at test they behaved similarly to juvenile and adult rats, suggesting that after early stress the role of $GABA_A$ receptors in extinction expression becomes more adult-like (77). These studies suggest that the development of fear retention and extinction learning, two behaviors with potential importance for vulnerability to mental health disorders (e.g., PTSD), are dynamically regulated by the early life rearing environment (see **Table 1** for a summary) and that stress is one condition under which increased vulnerability to mental health problems might emerge.

FGF2

Another early life event that has been shown to influence the development of fear learning and extinction is exposure to fibroblast growth factor-2 (FGF2). FGF2 is a neurotrophic factor that regulates cell proliferation, differentiation, and survival. During early development FGF2 is responsible for determining the overall morphology of the brain, and during adulthood it is released in response to stress or brain injury, potentially playing a neuroprotective role (78, 79). Early life exposure to FGF2 has

marked central effects; a single peripheral administration of FGF2 on P1 led to increased cell proliferation in the hippocampus, resulting in a larger hippocampal volume that was first evident at P4 and that persisted throughout adulthood (80). Conversely, transgenic mice that lack FGF Receptor 1 (the primary receptor for FGF2) have decreased hippocampal cell proliferation, resulting in permanent hippocampal atrophy (80, 81).

Graham and Richardson (82) investigated whether these long-term hippocampal morphological changes induced by early life exposure to FGF2 might lead to changes in hippocampal-mediated memory formation. They first examined the impact of early life FGF2 on contextual fear conditioning in the developing rat. Infant rats exhibit impaired long-term (i.e., after 24 h) memory for contextual fear relative to older rats (83). However, subcutaneous injections of FGF2 from P1-5 led to an early emergence of long-term memory for contextual fear in P16 rats. Early life FGF2 also enhanced contextual fear conditioning in P23 rats, an age at which rats exhibit moderate levels of long-term memory for contextual fear.

Graham and Richardson (82) then examined the impact of early life FGF2 on fear extinction at P16. In those studies, cued fear conditioning procedures were used (i.e., white noise CS paired with shock US) as infant rats can exhibit long-term memory of such associations. Animals were trained in one context, and then extinguished in a different context. Early life FGF2 did not affect the strength of cued fear conditioning, the rate of extinction acquisition, or the retention of extinction training when the extinguished CS was presented in the extinction training context. However, when the extinguished CS was presented in the original fear conditioning context, FGF2-treated P16 rats exhibited recovered fear responses whereas vehicle-treated P16 rats exhibited low fear responses. That is, early life FGF2 led to a precocious emergence of renewal. These results show that early exposure to FGF2 causes an accelerated emergence of the ability to encode and/or maintain a representation of the contextual elements associated with fear conditioning and extinction memories. When taken together with the findings from Cheng et al. (80) it is possible that these behavioral results are a consequence of the effects of early life FGF2 on hippocampal development.

The fact that FGF2, maternal-separation, and exposure to CORT have similar effects, all accelerating the development of fear learning in infant rats, raises the possibility that stress and FGF2 produce their outcomes on early fear learning and extinction through the same or a similar pathway. For example, it might be the case that FGF2 is one of the mechanisms involved in accelerated maturation following early stress. In support of this idea, a large body of evidence has suggested that FGF2 is critically involved in the effects of stress. FGF2 appears to be modulated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the mammalian response to stress. Adrenalectomized rats exhibit reduced expression of FGF2 in the hippocampus, striatum, and frontal cortex, whereas administration of glucocorticoids increases FGF2 mRNA in the hippocampus and prefrontal cortex; both results support the idea that adrenal hormones (which are responsible for terminating the stress response) exert control over FGF2 [see review by (84)]. Indeed, both physical and psychological stress upregulate FGF2. Specifically, brain injury leads to increases in FGF2 around the site of the lesion, and application of FGF2 to the lesion reduces cell death and increases astrocytic density (85, 86). Likewise, restraint stress (a psychological stressor) increases FGF2 mRNA expression in the hippocampus and prefrontal cortex (84). These findings point to a potential neuroprotective role for FGF2 in response to stress [see (79)].

There are several factors that determine whether or not FGF2 increases in response to stress, one of which is the controllability of the stressor. Bland et al. (87) exposed two groups of rats to a series of tail shocks. One group could terminate the shock by turning a wheel; the other group were yoked to the first and could not control the shock, but experienced the same number and intensity of shocks as the first group. Escapable, but not inescapable, shock led to a significant increase in hippocampal FGF2 protein expression 2 h post-shock, and this effect persisted for 24 h. Furthermore, inescapable shock, but not escapable shock, led to a significant decrease in the proliferation of hippocampal neural progenitor cells. A later study demonstrated that escapable shock, but not inescapable shock, also causes increases in FGF2 mRNA expression in the PFC (88). Similarly, Turner et al. (89) reported that chronic (4 days) social defeat stress, in which a rat is exposed to an aggressive male rat of a different strain, down-regulates hippocampal FGF2 mRNA expression. These findings suggest that endogenous FGF2 may protect against the harmful effects of stress (perhaps by increasing cell proliferation), but only if the animal has some level of control over the stressor.

Another factor that determines FGF2's involvement in the stress response is prior exposure to stress hormones. It has been shown that prenatal exposure to corticosterone significantly reduces basal FGF2 mRNA expression during adulthood. Furthermore, prenatal exposure to corticosterone significantly attenuates the upregulation of hippocampal FGF2 mRNA normally seen following acute stress in adulthood (84). Therefore it is possible that early life stress may alter (i.e., cause dysfunctions in) FGF2's neuroprotective response to stress later in life (79).

HOW DO EARLY STRESS AND FGF2 EXPOSURE ACCELERATE THE DEVELOPMENT OF FEAR LEARNING SYSTEMS?

One intriguing possibility concerning the effects of stress and FGF2 exposure on accelerated emotional development is that these early experiences regulate the expression of critical period plasticity. Specifically, it is possible that infantile amnesia, impaired context learning, and relapse-resistant extinction represent forms of critical period plasticity in emotional systems, and that these forms of plasticity are controlled by the same cascade of signals as critical periods in other areas of the brain. That is, stress exposure could initiate a cascade of cellular and molecular changes involved in terminating infant-like forms of fear learning via HPA activation of FGF2 receptors. This would be an attractive, and simple, explanation for the similar outcomes of early stress and FGF2 exposure on developmental transitions in fear learning. In other words, it is possible that stress and FGF2 activate a "signature" set of signals involved in critical period termination across the brain.

Traditionally, critical/sensitive periods have been defined as discrete stages of rapid neural development in which plasticity is enhanced, allowing early environmental input to fine-tune final wiring patterns in the brain before plasticity is reduced in

adulthood [e.g., (90–92)]. The onset and offset of critical periods is not a simple age-dependent maturational process. Rather, the timing of critical periods can be manipulated by different experiences which affect the various molecular and cellular signals involved in their opening and closure (90). While the high levels of plasticity inherent in a critical period allow for enhanced learning and refinement of neural functions these periods also represent a time of vulnerability for the developing brain. If aberrant sensory or social events are experienced, or expected environments do not manifest, then the timing and function of the critical period can be altered, placing the brain at risk for abnormal wiring patterns and adverse behavioral/sensory outcomes. For instance, some developmental disorders in humans (e.g., autism) have been proposed to result from a disruption in the timing or expression of critical periods across various brain regions (93, 94).

There are many different critical periods which occur across development, each involving unique brain regions or neural circuits (95). For example, critical periods in humans have been proposed for the development of sensory/sensory-motor, cognitive, and emotion systems [e.g., (4, 7, 96, 97)]. For instance, when learning takes place before the age of 7 years, acquisition of a second language usually occurs to a level that is grammatically indistinguishable from that of native speakers. However, mastery of a second language becomes progressively harder from 8 years onward (98, 99). Other research has shown that children need to be exposed to appropriate levels of cognitive, tactile, and emotional stimulation early in life in order to develop adequate cognitive functions and emotion regulation skills. Children reared in institutional settings which lack the appropriate levels of stimulation exhibit profound deficits in cognitive and emotional development, effects which are often permanent if children are not adopted before the age of 2 years [see (3), for a review; (100)].

In non-human animals critical/sensitive periods have also been shown to occur in a variety of sensory and emotional systems, such as song learning in birds, attachment learning in rats, and cortical responses to vibrissa stimulation in rats [see (101–103), for a review; (71, 104, 105)]. The best characterized animal model of critical period plasticity, however, is that of ocular dominance (OD) plasticity induced by monocular deprivation [(106, 107); see (91, 108), for reviews; see also (92), for a review]. Only during the critical period for OD plasticity does closure of one eye result in a loss of visual acuity in the closed eye (amblyopia) and a shift in the responsiveness of neurons in the primary visual cortex away from the closed eye.

Research investigating OD plasticity has highlighted numerous molecular and cellular signals which are involved in opening and closing this critical period. Importantly, these signals have been shown to regulate the timing of sensitive periods in other sensory modalities (104, 105), suggesting that there may be a general neural signature which guides critical period timing across the brain. Although the neural signature for critical period timing has mostly been investigated in sensory systems, recent evidence suggests that the same signals may also regulate sensitive periods in fear learning (46). Further, there is some evidence to suggest that those neural signals are regulated by particular types of early experience, suggesting a potential mechanism via which stress/CORT/FGF2 may have affected the timing of adult-like fear retention, context learning, and extinction described earlier. While a detailed analysis of the molecular and cellular events involved in triggering the onset and offset of critical period plasticity in the visual cortex is beyond the scope of this review [interested readers are referred to excellent past reviews on the topic: (91, 92, 108)], we provide a brief summary of those molecular and cellular signals important for critical period plasticity in the visual system that also may have a role in fear and extinction learning and that appear to be regulated by stress/CORT/FGF2.

SIGNALS INVOLVED IN THE OPENING OF CRITICAL PERIOD PLASTICITY

The onset of OD plasticity appears to be triggered by a change in the balance of excitation and inhibition in the visual cortex, mostly as a result of developmental increases in inhibitory activity. For example, 4 days of monocular deprivation starting on P25–P27 induces OD plasticity in wild-type mice but not in mice with a genetic knockout (KO) of the GAD65 gene, which inhibits GABA release. However, critical period plasticity could be rescued in GAD65 KO mice if levels of inhibition were artificially increased during monocular deprivation via infusion of a benzodiazepine directly into the visual cortex (109). Benzodiazepines were also successful in precociously inducing OD plasticity when monocular deprivation was performed in pre-critical period mice [P15–P20; (90)]. It appears that the maturation of intra-cortical inhibition is regulated by brain derived neurotrophic growth factor (BDNF) because genetic over-expression of BDNF across postnatal development accelerated the maturation of parvalbumin positive (PV+) GABAergic interneurons in the visual cortex and resulted in a precocious critical period (110, 111). Together these studies suggest that the molecular machinery for enhanced plasticity is present early in life but that maturation in GABAergic circuitry (e.g., PV+ interneurons and GABAergic synapses) pushes inhibitory activity beyond a certain threshold to trigger the opening of the critical period.

SIGNALS INVOLVED IN THE CLOSURE OF CRITICAL PERIOD PLASTICITY

While intra-cortical inhibition appears to be sufficient for the initiation of critical period plasticity, there are several mechanisms that appear to be involved in critical period termination, many acting as "structural brakes" which limit plasticity. For example, critical periods appear to be regulated by the appearance of extracellular matrix proteins – perineuronal nets (PNNs) – around the dendrites, axons, and cell bodies of GABAergic neurons. PNNs are believed to limit critical period plasticity by increasing stability of synapses via inhibition of axonal growth and sprouting. Appearance of PNNs in various brain regions correlates with termination of critical period plasticity in several different sensory systems [e.g., (92, 104, 105, 108)], and recently appearance of PNNs in the amygdala was shown to correlate with the termination of infant-like, relapse-resistant extinction learning and the transition into adult-like, relapse-prone extinction learning (46). Interestingly, when PNNs in the visual cortex or amygdala of adult rats are degraded via chondroitinase ABC (chABC), then the critical periods for OD plasticity and erasure-like extinction, respectively, are reopened (46, 112). This research strongly suggests that erasure-like extinction represents a form of critical period plasticity occurring in emotion circuits in the brain, and that

termination of this form of infant plasticity appears to be regulated by some of the same structural brakes as critical period plasticity in sensory systems.

In addition to the formation of PNNs, other developmental factors also appear to be involved in limiting structural plasticity in the visual cortex and terminating the critical period for OD plasticity. For example, maturation of myelin basic protein (MBP) in the visual cortex has been shown to correlate with termination of the critical period for OD plasticity (113), potentially through inhibiting mechanisms of structural remodeling necessary for plasticity (114). The myelin associated growth inhibitor Nogo-66 is known to limit axonal regeneration following CNS damage because antagonizing the Nogo-66 receptor (NgR) promotes axonal regeneration following spinal cord injury in the rat (115). Interestingly, when the NgR was genetically deleted in mice and monocular deprivation occurred post-sensitive period (i.e., at P45) the mutant mice exhibited OD plasticity whereas wild-type mice did not (113). Hence, it appears that adult mice retain the capacity for enhanced plasticity but that increased myelination in the visual cortex which occurs across development acts as a structural brake, limiting OD plasticity.

Another factor that has been implicated in the closure of the critical period for OD plasticity is calcium/cAMP response element binding protein (CREB)-mediated gene transcription. Evidence for the role of CREB activity in OD plasticity comes from studies which have shown that monocular deprivation during the critical period stimulates CREB-mediated gene transcription whereas post-critical period monocular deprivation has a less pronounced effect on CREB-mediated processes (116). Further, when CREB activity is enhanced in adult mice (through the use of a transgenic mouse line expressing VP16-CREB, which leads to constitutively active CREB across life), it has been shown that persistent OD plasticity can be induced in the visual cortex (117). Also, inhibiting upstream regulators of CREB (e.g., PKA) in cats decreases OD plasticity during the critical period (118).

It has been proposed that CREB is important in terminating critical periods because it regulates the activity of plasticity-modulating genes (92). Studies examining candidate CREB-mediated genes that might be involved in OD plasticity have focused on micro RNA (mir) 132 which has been implicated in neural plasticity (119). In a recent study, increasing mir132 expression in mice before monocular deprivation blocked OD plasticity during the critical period (120), suggesting that mir132 acts as a brake on plasticity.

STRESS, CORTICOSTERONE, AND FGF2 REGULATE MOLECULAR AND CELLULAR SIGNALS INVOLVED IN CRITICAL PERIOD TIMING

Early exposure to stress, corticosterone, and FGF2 has been shown to accelerate the transition into adult-like fear retention and extinction learning in infant rats; early exposure to those events led to a precocious termination of the critical period for infantile amnesia and erasure-like extinction. It is possible that stress/CORT/FGF2 exposure hastened the developmental transitions in fear learning by acting on those processes known to be involved in critical period regulation in other systems. Indeed, evidence shows that early life adversity, CORT, and FGF2 regulate many of the molecular and cellular signals involved in both the opening and the closure of critical periods, accelerating the developmental emergence of those signals in brain regions important for emotional responding in adults. However, those rodent studies which examined environmental regulation of infant fear retention and extinction only measured outcomes at one time point making a determination of the early closure of the critical period possible but determination of an early opening of the critical period uncertain. It could be the case that the critical period for infantile amnesia and erasure-like extinction opened at the same time in MS/CORT/FGF2 and SR/vehicle rats, but that this period closed earlier in the MS/CORT/FGF2 rats (i.e., the time frame for the critical period was compressed). Alternatively, it may be the case that MS/CORT/FGF2 led to an early opening as well as an early closure of the critical period (see **Figure 1** for a depiction of these possibilities). The fact that stress and FGF2 appear to regulate signals involved in both the opening and closure of critical periods, however, suggests that the latter case is most likely the case (i.e., that stress/FGF2 leads to an early opening and closure of the critical period in fear learning).

Evidence that stress might regulate critical period opening in the emotional system comes from studies examining the effect of early stress on GABAergic development. Specifically, maternal-separation has been shown to lead to a more mature

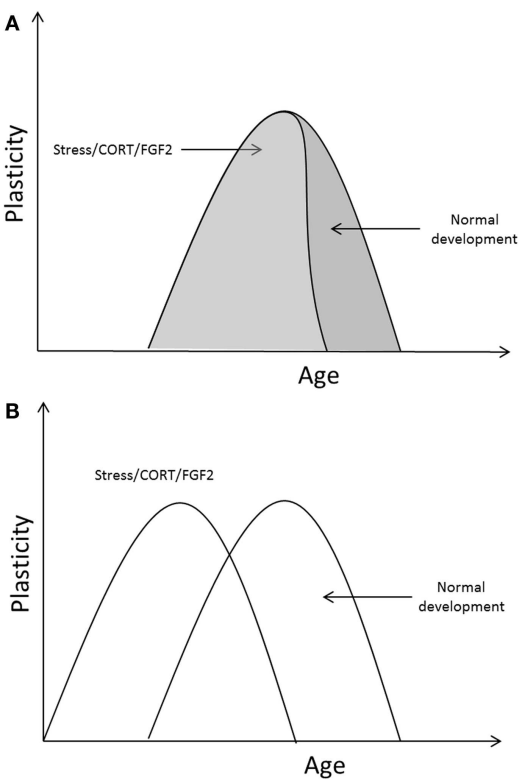

FIGURE 1 | Two potential outcomes of the effect of stress/CORT/FGF2 on critical period timing in the emotion system. **(A)** Different manipulations may alter the duration of the critical period but may not affect the age at opening. **(B)** Once opened, the duration of the critical period may be relatively static; manipulations causing an early opening of the critical period in emotional plasticity would also cause an early closure.

form of GABAergic signaling in the CA1 region of the infant hippocampus of male rats (121), and maternally separated rats also exhibit a short-term upregulation of BDNF in the PFC and hippocampus at P17 (122). As mentioned earlier, the critical period for OD plasticity is triggered by maturation of GABA in the visual cortex. Further, early over-expression of BDNF in the visual cortex was shown to accelerate GABAergic maturation and lead to a precocious emergence of OD plasticity.

In addition to influencing signals involved in the opening of critical periods, early life stress/corticosterone/FGF2 also appear to regulate some of the structural brakes on plasticity. For example, early life stress (caused by weaning rats at P14 rather than P21) has been shown to accelerate whole-brain, as well as amygdala-specific, myelination in P21–P35 male mice (123, 124). Also, elevated glucocorticoids have been shown to accelerate the initiation and rate of myelination in co-cultures of Schwann-cell and neurons taken from infant rats (125). In addition, oligodendrocyte cells express FGF receptors, and FGF2 application to cultured cells stimulates proliferation of oligodendrocyte precursor cells (126). FGF2 has also recently been identified as a critical regulator of myelin sheath thickness. Furusho et al. (127) created a line of mutant mice that lacked the FGF receptors 1 and 2, the two receptors to which FGF2 binds. They reported that while mutant mice exhibited normal initiation of myelination in the spinal cord at P4 (as judged by immunoblotting for MBP), by P30 mutant mice exhibited significantly less MBP positive myelin, and reduced overall white matter area, compared to control mice, suggesting a reduction in myelin synthesis. Accordingly, while myelin thickness increased from P15 to 10 months of age (the oldest age tested) in control mice, myelin thickness stalled in mutant mice, who exhibited thinner myelin compared to control mice from PND 30 to 10 months of age. Importantly, the numbers of myelinated and unmyelinated axons was comparable in control and mutant mice at all ages tested, suggesting that FGF2 plays a specific role in signaling for the development of myelin thickness. Hence, it is possible that early life exposure to FGF2, stress, or to stress hormones may help to precociously terminate critical periods in fear learning via accelerating the rate of myelin development in the hippocampus, amygdala, and mPFC.

Along with potentially accelerating structural brakes in plasticity, it is also possible that early life stress/CORT/FGF2 exposure caused an early termination of infantile amnesia, impaired context learning, and erasure-like extinction via a CREB-mediated pathway. For example, many of FGF2's neurotrophic effects appear to be mediated by phosphorylation of CREB. Sung et al. (128) showed that FGF2 increases hippocampal neuronal differentiation and outgrowth via causing phosphorylation of CREB and CRE-mediated gene transcription. They also demonstrated that FGF2-induced neuronal outgrowth was blocked in cells that contained a dominant negative CREB construct (blocking CREB activation). FGF2 also appears to regulate hippocampal cell proliferation via phosphorylation of CREB (129), and FGF2-induced cell proliferation is blocked by a CREB inhibitor. Cell proliferation was markedly increased in cell cultures that over-expressed CREB, but only if FGF2 was applied to these cultures. In other words, CREB over-expression did not increase cell proliferation by itself, suggesting that FGF2 recruits CREB to increase cell proliferation. In addition, recent research has shown that early exposure to stressors (e.g., maternal-separation) regulates the expression of non-coding RNAs which are mediated by CREB. Specifically, Uchida et al. (130) showed that MS180 from P2 to P14 increased the expression of mir132 in the PFC of P14 mice relative to SR P14 mice. Furthermore, FGF2 has been shown to upregulate mir132 in cultured immature cortical neurons, as well as in cultured astroglial cells (131). As mentioned earlier, alterations in the expression of mir132 have been shown to regulate critical period timing for OD plasticity.

Together the findings just reviewed suggest that early life exposure to FGF2/stress/CORT may regulate the developmental timing of critical periods in fear learning via accelerated maturation of BDNF expression, GABAergic inhibition, myelination, and CREB-mediated gene transcription in those brain regions critical for fear memory and extinction learning in adults – the hippocampus, mPFC, and amygdala (see **Figure 2**). If this were true, it would

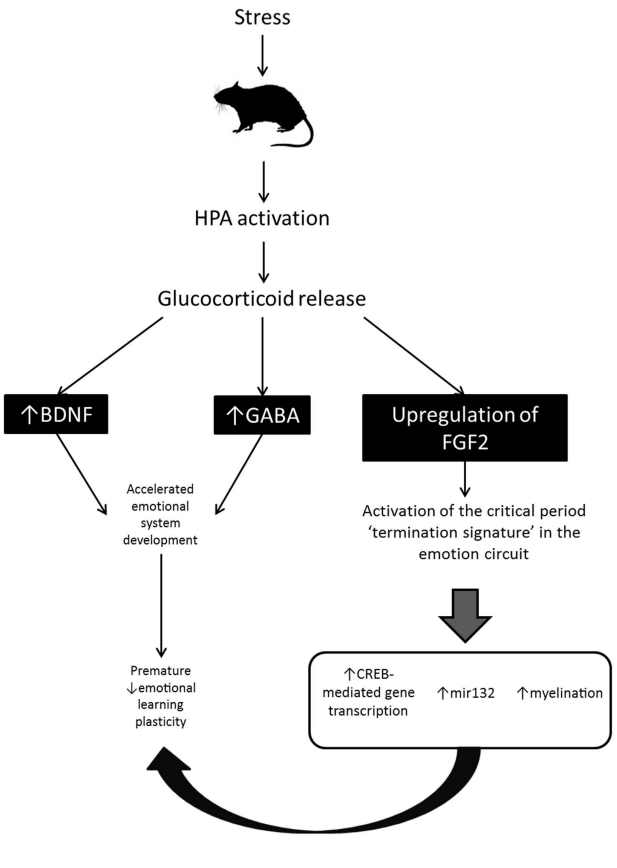

FIGURE 2 | Proposed mechanism by which chronic stress accelerates the developmental transition between infant and adult-like forms of fear retention and fear learning in rodent models. Stress-induced activation of the HPA axis results in increased BDNF and GABA, and central upregulation of FGF2. BDNF and GABA stimulate early development of the emotion system and may lead to early opening of the critical period for infantile amnesia and erasure-like extinction. FGF2 upregulation triggers activation of the critical period "termination signature" in the emotion circuit (i.e., activates the cellular and molecular mechanisms known to be involved in critical period timing in sensory systems). Activation of those signals leads to an early termination of fear learning plasticity.

support the idea that there may be a common neural signature which guides critical periods of plasticity across the brain. This idea has been raised by previous researchers to explain the finding that the same molecular and cellular signals appear to be involved in a variety of critical periods in different sensory systems (108). However, the idea that the same molecular and cellular signals may regulate critical periods of plasticity for fear learning in subcortical circuits (e.g., the amygdala) has only recently begun to be explored [e.g., (46)].

POTENTIAL FOR MULTIGENERATIONAL EFFECTS OF STRESS/FGF2 ON FEAR LEARNING

If critical period mechanisms are involved in regulating the opening and closure of fear learning plasticity then a potential implication is that the effects of environmental manipulations on maturation of fear learning might be heritable. Indeed, epidemiological evidence suggests that the effects of stress on mental health can be transmitted across multiple generations. For instance, mothers that were exposed to the September 11 terrorist attacks in New York City during pregnancy and who subsequently developed PTSD were shown to exhibit a suppressed basal cortisol response (132). Interestingly, a similar profile of cortisol suppression was also evident in the infants of those mothers, with that being especially true for infants of mothers that were in the third trimester of pregnancy when the attacks occurred. In addition, high risk phenotypic traits for mental health problems (e.g., behavioral inhibition) have been shown to exhibit a high degree of heritability, which can be attributed to both genetic and environmental factors (133–136). Hence, there is clear epidemiological data suggesting that mental health disorders and the influence of stress on the emergence of those disorders is heritable.

Animal models have been increasingly used to investigate the intergenerational transmission of neurobehavioral alterations after stress (137–140). Several studies in rodents have shown that stress-evoked alterations in parenting style are passed onto offspring, and that these behavioral alterations are often accompanied by neuroendocrine changes (141). In addition, epigenetic modifications to gene transcription caused by early life stress have been shown to persist across the life of the rat and to be passed onto biological offspring (137). Interestingly, more recent studies have demonstrated that actual stress-induced behavioral phenotypes can also be transmitted across generations. For example, maternally separated rats exhibited depressed behaviors as adults, and these same depressive behaviors were also exhibited by their adult offspring and grandchildren, despite those subsequent generations never being exposed to stress (138). Hence, animal research has been useful in modeling the transmission of both neurological as well as behavioral alterations caused by stress. One currently unexplored possibility is that stress-induced alterations to the maturation of fear retention and extinction systems could also be transmitted to subsequent generations. Indeed, some of the mechanisms involved in critical period opening and closure could potentially lead to such a transgenerational profile. Specifically, research has shown that transgenerational effects can be produced by alteration of cytoplasmic RNAs (e.g., miRNA), which can be carried in the sperm and eggs and can epigenetically alter the phenotype of subsequent offspring. Recently it has been proposed that miRNAs may be important in the transmission of environmentally induced phenotypic changes across generations because some RNAs can survive degradation during embryogenesis and have been shown to regulate offspring phenotype [e.g., (142)]. The evidence for this comes from experiments which show that injection of a miRNA critical for brain development [mir124; (119, 143)] directly into cell embryos resulted in offspring which exhibited a much faster growth rate (increased by 30%) than non-injected offspring (144). Importantly, this "giant" phenotype was transmitted across multiple generations via alterations of mir124 in the spermatozoa. Hence, changes in the expression levels of certain miRNAs can be incorporated into the germ-line of animals and produce a transgenerational phenotype. As mentioned earlier, a recent study showed that a miRNA important for inhibiting OD plasticity in the visual cortex (mir132) and the miRNA which produced a transgenerational "giant" phenotype (mir124) was upregulated in the mPFC of P14 mice following maternal-separation (130). Further, mir132 is upregulated by FGF2 (131). Hence, it is possible that the expression of these miRNAs may regulate critical period closure in fear learning systems and that stress/FGF2-induced alterations in these miRNAs could be heritable. Such hypotheses will need to be investigated in future studies.

BRIDGING THE GAP BETWEEN BASIC AND CLINICAL WORK: CLINICAL IMPLICATIONS AND POTENTIAL TRANSLATION OF STRESS/FGF2-INDUCED ACCELERATION OF EMOTIONAL DEVELOPMENT IN ANIMAL MODELS

The fact that infantile amnesia and relapse-resistant extinction are regulated by stress, FGF2, and potentially other early life events is highly relevant for clinical researchers working on understanding and treating mental health disorders across the lifespan. Early life stress is one of the greatest contributing risk factors for mental health problems across all life stages (145), relating not only to risk for mental health disorders but also to transdiagnostic features common of many psychological disorders [e.g., increased emotional reactivity; (146, 147)]. Further, early adversity and abuse has been shown in human populations to interact with specific genetic polymorphisms to predict adult major depressive disorder and PTSD (148, 149). However, the developmental trajectories which are altered by such gene × environment interactions remain elusive. The body of research reviewed in this paper suggests that early emerging changes in fear learning and extinction resulting from stress may be one outcome which could affect emotional responding across the lifespan and which might interact with genetics to produce stable phenotypes of risk for mental health disorders. For example, it is possible that stress exposure during a critical period of development early in life paired with a later experienced trauma might lead to a phenotype of treatment-resistant PTSD in genetically predisposed individuals via a pathway of altered development of the fear extinction system; such a possibility should be examined in future studies.

The possibility that infantile amnesia and relapse-resistant extinction may represent critical period plasticity in fear learning also has significant clinical implications, especially when considering potential pharmacological treatments for mental health disorders. As discussed earlier, the involvement of critical period

molecular signals in terminating fear learning plasticity opens up a possible mechanism via which the effects of stress/FGF2 exposure might increase vulnerability for mental health problems across multiple generations. In addition, they also suggest several novel mechanisms via which anxiety disorders and other mental health problems might be treated. Specifically, if critical periods of emotional learning could be reopened in adulthood (or at any point after they have closed) it may help treat the root of many anxiety disorders (i.e., persistent expression of fear and relapse after extinction). In other words, it is possible that anxious individuals might be treated with pharmacological adjuncts to reopen infant-like forgetting and relapse-resistant extinction, which could then be combined with therapy to improve treatment efficacy. Indeed, there have been three recent studies which suggest that the critical period of erasure-like (relapse-resistant) extinction can be reopened in juvenile and adult rats. The first evidence that relapse-resistant/erasure-like extinction could be reactivated in adult rats came from Gogolla et al. (46). In those studies appearance of PNNs around GABAergic amygdala interneurons was correlated with the natural transition from relapse-resistant extinction in infant mice to relapse-prone extinction in juvenile mice. That is, at the same time that rats began to exhibit relapse behaviors after extinction there was a significant increase in the number of PNNs in the amygdala. To examine whether the formation of the PNNs was sufficient to cause the transition into adult-like extinction Gogolla et al. degraded amygdala PNNs with chABC in adult mice before conditioning. The treatment with chABC significantly reduced the number of PNNs in the adult amygdala and also reduced the expression of relapse behaviors after extinction (i.e., the chABC-treated adults did not show renewal or spontaneous recovery of extinguished fear). Hence, it appears that the infant profile of extinction learning could be reactivated in adulthood by removal of one of the structural brakes on plasticity – PNNs.

Another line of evidence that "erasure-like" extinction can potentially be activated in adult rats comes from recent work on the impact of acute, exogenous FGF2 on extinction of conditioned fear (150–153). Those studies demonstrated that systemic or intra-amygdala infusion of FGF2 not only enhanced extinction in juvenile and adult rats, but it also significantly reduced renewal and reinstatement, even when vehicle-treated rats were given four times the amount of extinction training to match extinction strength between vehicle- and FGF2-treated groups. In other words, when treated with FGF2, adult rats exhibit the behavioral qualities of infant-like (erasure-like) extinction. The neurobiological mechanisms by which FGF2 causes infant-like extinction are unknown. Nevertheless, similar to findings in the visual system, it appears that adult rats retain the capacity for infant-like extinction and that this form of plasticity can be reactivated rapidly under conditions which favor that plasticity.

In order to investigate the possibility that extinction combined with FGF2 leads to an erasure of the original fear memory, Graham and Richardson (152) exploited recent findings regarding re-extinction, which refers to the process of relearning extinction following reacquisition of fear to an extinguished cue. Converging evidence strongly suggests that whereas initial extinction in adult rats is impaired by NMDAr antagonists, re-extinction is not impaired by NMDAr antagonists (154–157). This suggests that relearning to extinguish fear does not depend on NMDAr activity. However, Graham and Richardson (152) found that when rats were systemically injected with FGF2 immediately after extinction training, then retrained to fear the extinguished CS, and then re-extinguished following treatment with an NMDAr antagonist, FGF2-treated rats exhibited impaired re-extinction retention. In contrast, rats that were extinguished with vehicle and then re-extinguished following treatment with an NMDAr antagonist did not exhibit any impairment in re-extinction retention. That is, during re-extinction FGF2-treated rats "behaved" as if the CS was being extinguished for the first time. Interestingly, similar results have been obtained for juvenile rats that are extinguished to a CS at PND 16 (during the "erasure-like extinction" period of development), and then retrained and re-extinguished to the same CS later in development. In this instance, re-extinction is also NMDAr-dependent (158). Together, these findings suggest that FGF2 treatment, when combined with extinction training, may reactivate the "erasure-like" fear extinction observed in infant rats.

The third study to attempt to reactive infant-like plasticity in rodents during extinction learning was performed by Karpova et al. (159). In that study adult mice were chronically exposed to the antidepressant fluoxetine in their drinking water either before or after fear conditioning and during extinction and test. They showed that the fluoxetine-exposed mice behaved like infant mice in past studies (46), showing less post-extinction relapse than the vehicle-treated mice. In addition, fluoxetine treatment also resulted in a lower proportion of PNNs in the BLA, suggesting that the effect of fluoxetine on relapse behaviors after extinction may have occurred through facilitating the removal of structural brakes on plasticity (PNNs). Interestingly, combining antidepressant treatments like fluoxetine with exposure therapy in humans has often yielded better results than either treatment alone (160). The study by Karpova et al. (159) suggests that fluoxetine-induced reactivation of the critical period for erasure-like extinction might underlie those clinical findings.

CONCLUSION

The findings regarding accelerated development of fear learning by stress/CORT/FGF2 are theoretically relevant because they demonstrate that the rate at which particular forms of learning and memory mature across the lifespan can be influenced by a range of early life experiences. Until recently, no one had examined how early experiences affected fear retention and extinction development, despite these forms of emotional learning being critically involved in the pathogenesis and treatment of mental health problems. The studies reviewed here show that the timing of the maturation of fear learning is not set in stone but can be dynamically regulated by early experience. In addition, these findings are clinically relevant because early life adversity is a common feature in persons with psychopathology [e.g., (161, 162)], and fear retention and extinction in rats are important pre-clinical models of anxiety problems in humans (10, 163, 164). Although many theories have suggested that early experiences are critical for the emergence of anxiety and other mental health problems in humans (165–168), no studies, until very recently, had examined how fear retention and extinction are impacted by different early experiences in infant rodents. In addition, within the human literature,

there are reports of individual differences in the processes of fear retention and extinction which may underlie subsequent vulnerability to develop anxiety problems [e.g., (169, 170)], yet there is little information on what factors might influence those differences or the molecular mechanisms which might underlie them.

While the findings regarding environmental alteration of the maturation of fear learning systems are novel, at this stage there are no definitive answers about what molecular and cellular mechanisms drive the normal development of these emotion systems, nor the accelerated transition produced by stress/CORT/FGF2. However, the fact that all three manipulations have a similar effect on emotion system development, that stress/CORT regulate FGF2, and that stress/CORT and FGF2 appear to regulate some of the signals involved in critical periods of plasticity in sensory systems hints at a potential mechanism for transitions in fear learning. Specifically, we have suggested that the expression of infantile amnesia and relapse-resistant extinction in infancy may represent critical period plasticity and propose a model in which early environments that alter the age at which the developmental transitions occur (e.g., stress) might function through an HPA/FGF2-dependent activation of "critical period signals," in turn leading to an early termination in emotional plasticity (see **Figure 2** for a graphical depiction of this model). The proposed model, although speculative, does suggest some potential avenues for future research. Specifically, if the principles guiding critical period plasticity in sensory systems can also be generalized to emotion learning, it should be possible to manipulate the timing of infantile amnesia and erasure-like extinction via alteration of any of the signaling pathways involved in critical period plasticity in sensory systems. Also, interfering with any of the signaling pathways involved in critical period plasticity should change the effect of stress on fear retention and extinction learning in infant rats. One possibility, for example, might be to chronically suppress levels of BDNF while rats are experiencing maternal-separation to determine whether accelerated emergence of adult-like fear retention and extinction still occurs. All these possibilities would have important outcomes both theoretically, in understanding the guiding principles of critical period plasticity, as well as clinically, in understanding how particular experiences might impact emotional development across the life span. Although these speculations require further examination, the reviewed literature is clearly developing a foundation for examining the experience-dependent modulation of critical period opening and closure in emotional systems, an area with significant implications for our understanding and treatment of anxiety disorders (e.g., PTSD).

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Regulating critical period plasticity: insight from the visual system to fear circuitry for therapeutic interventions

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Early temporary windows of heightened brain plasticity called critical periods developmentally sculpt neural circuits and contribute to adult behavior. Regulatory mechanisms of visual cortex development – the preeminent model of experience-dependent critical period plasticity-actively limit adult plasticity and have proved fruitful therapeutic targets to reopen plasticity and rewire faulty visual system connections later in life. Interestingly, these molecular mechanisms have been implicated in the regulation of plasticity in other functions beyond vision. Applying mechanistic understandings of critical period plasticity in the visual cortex to fear circuitry may provide a conceptual framework for developing novel therapeutic tools to mitigate aberrant fear responses in post traumatic stress disorder. In this review, we turn to the model of experience-dependent visual plasticity to provide novel insights for the mechanisms regulating plasticity in the fear system. Fear circuitry, particularly fear memory erasure, also undergoes age-related changes in experience-dependent plasticity. We consider the contributions of molecular brakes that halt visual critical period plasticity to circuitry underlying fear memory erasure. A major molecular brake in the visual cortex, perineuronal net formation, recently has been identified in the development of fear systems that are resilient to fear memory erasure. The roles of other molecular brakes, myelin-related Nogo receptor signaling and Lynx family proteins – endogenous inhibitors for nicotinic acetylcholine receptor, are explored in the context of fear memory plasticity. Such fear plasticity regulators, including epigenetic effects, provide promising targets for therapeutic interventions.

Keywords: critical period, visual cortex plasticity, fear erasure, perineuronal nets, lynx1, HDAC inhibitors, reconsolidation update

INTRODUCTION

As the brain develops, particular regions undergo different critical periods of plasticity when their underlying circuits gain heighted sensitivity to experience (1, 2). Experience during these early temporal periods has a profound effect on the wiring of skills and behaviors, such as language, music playing, visual processing, and emotional processing. When the critical period for a region closes, the adaptations in its circuitry become fixed, locking in adjusted ways of processing and responding to stimuli and bringing plasticity into a latent state. This mechanism is normally a beneficial way to retain optimized behaviors without need for maintenance or renewal. However, in individuals exposed to inappropriate stimuli, adaptive changes that were helpful during this window of developmental plasticity may not be beneficial in the future and can lead to dysfunctional behavior. Understanding the mechanisms that open and close critical period development can inform interventional strategies that attempt to modify these pathways later in life. In this review, we turn to the visual cortex as a well-developed model of experience-dependent critical period plasticity to provide novel insights for the mechanisms regulating plasticity in the fear system.

CRITICAL PERIODS ACROSS BRAIN FUNCTIONS

CRITICAL PERIOD FOR VISUAL CORTEX PLASTICITY

The visual system offers valuable insight through the study of critical period mechanisms. In humans and animals, visually depriving one eye by obstructing it early in life yields loss in visual acuity (amblyopia) by stimulating an anatomical remodeling within primary visual cortex (3). Importantly, such an effect of visual deprivation has not been seen in the adult, strongly suggesting the presence of a developmental critical period for visual experience-dependent plasticity in visual cortex. Due to a lack of sufficient brain plasticity in adulthood, untreated monocular deprivation during childhood results in life-long amblyopia, a condition affecting 2–5% of the human population (4). Indeed, recovery from deprivation amblyopia in adulthood is limited across species, from higher mammals (3), to rodents (5, 6), and requires therapeutic intervention. Over the last 10 years, the murine visual

system, has emerged as a valuable model system for creating such intervention, having a well-defined, 2 week critical period that peaks 1 month after birth (**Figure 1A**). The predictability and duration of this temporal window is particularly useful for dissecting the molecular mechanisms of visual cortex plasticity through genetic manipulation and environmental intervention (7). Critical period mechanisms identified in rodent visual cortex have not only catalyzed multiple pharmacological and behavioral interventions that aid functional recovery in adults (8), but have also guided research uncovering molecular mechanisms of critical period plasticity in other brain regions, especially the auditory and fear systems (9–11).

CRITICAL PERIOD FOR FEAR MEMORY ERASURE

Evidence from both animal and human studies suggest that the pathways underlying fear systems also undergo age-related changes in experience-dependent plasticity. Such age-related changes have been observed in both fear memory acquisition and extinction. Fear acquisition, measured through the ability to develop conditioned context-shock fear responses, does not emerge until 13–14 days (12). However, the mechanism governing the age-dependent change is little explored. Developmental differences in fear extinction, characterized by the ability to re-encode a previously encoded fear response, are observed in human studies and translate to rodent models, providing an effective animal model for exploring fear circuitry plasticity mechanisms. Fear extinction may be temporary, or it can lead to permanent fear memory erasure. The outcome of fear extinction is age-dependent. Fear extinction during a critical period in preadolescent mice (P13-17), leads to permanent fear erasure (13, 14). On the other hand, mice extinguished 24 days after birth or later exhibit a returned fear response (15) (**Figure 1B**). The juvenile form of fear extinction is also marked by an accelerated rate of change compared to that of adult mice (16), but the mechanistic relationship between the persistence and rate of fear extinction is unknown. Current evidence also suggests that erasure is specific to early temporal windows following a traumatic event (1–3 days later), as rodents undergoing later extinction training exhibit long-term hyper-vigilance rather than complete erasure (17). Understanding the mechanisms underlying these age-related changes in fear system plasticity may contribute to the better understanding of fear disorders, such as post traumatic stress disorder (PTSD), that are characterized by the re-experiencing of the traumatic events, hyper-vigilance, and persistent dysfunctional wiring of fear circuitry (18, 19).

PARALLELS BETWEEN VISUAL AND FEAR SYSTEM PLASTICITY

To what extent are visual and fear system critical period mechanisms parallel? Both the visual and fear systems maintain a developmentally limited ability to rewire connections that are no longer functionally appropriate. In the visual system, critical period (but not adulthood) monocular deprivation triggers a functional re-adaptation via visual cortex restructuring that reduces the input from the visually deprived eye. Early development fear extinction modifies behavior by erasing fear memories that are no longer appropriate, and differs from adult extinction that is temporary and slower. Thus, to discuss the role of molecular regulators in critical periods of plasticity, we consider them within their functional contexts and in functional parallels between systems.

To interrogate the extent to which the regulatory mechanisms of critical period plasticity in the visual system apply to the fear system, we conceptually consider "permanent fear erasure" analogously to "amblyopia," both of which are only induced during critical period. Accordingly, we also consider "fear extinction procedure" in fear system and "monocular deprivation" in the visual system as the inducers of plasticity in each system. Because the juvenile form of fear extinction is also marked by accelerated changes in response compared to that of adult mice (16), we consider both the rate and persistence of fear memory extinction as the measures of fear plasticity. Although the following discussion focuses on this parallel nature of plasticity between visual and fear system development, it should be noted that additional mechanisms likely contribute to the age-related changes described, including fear memory erasure due to increased rates of neurogenesis during development (20).

MOLECULAR BRAKES: COMMON MECHANISMS FOR CRITICAL PERIOD CLOSURE?

Recent studies using rodent visual cortex have identified multiple structural and functional molecular "brakes" that actively limit plasticity and close the critical period in the adult brain (8, 21). Structural brakes include PNNs (22), myelin-related inhibitory signaling mediated by Nogo receptor (23), and paired immunoglobulin-like receptor B expression (PirB) (24). Functional brakes, such as the nicotinic receptor binding protein Lynx1 act upon excitatory-inhibitory balance within local circuits (25). Importantly, lifting these brakes can induce critical period plasticity in adulthood and re-introduce ocular dominance remodeling following monocular deprivation. Here, we consider the potential roles of these major brakes explored in the visual system within fear circuitry.

PERINEURONAL NETS

Perineuronal nets are extracellular macromolecular aggregates associated with several subclasses of chondroitin sulfate proteoglycans (CSPGs) that surround neuronal cell bodies and proximal dendrites (26). In the visual system, PNNs inhibit experience-dependent plasticity observed during the critical period (**Figure 1A**). Further organization of CSPGs into PNNs coincides with the end of the critical period. Interestingly, PNN degradation with chondroitinase-ABC, an enzyme that degrades a key linkage glycoprotein and attacks CSPG side chains that allow the aggregates to form, restores experience-dependent plasticity in adult rats (22). Mice lacking a cartilage link protein that attenuates PNNs, Crtl1, consistently retain juvenile levels of plasticity in the adult visual cortex (27). Further, chondroitinase-ABC treatment coupled with reverse lid-suturing in adult rats – opening the sutured lid of the visually deprived eye while suturing the lid of the other, visually active eye – causes a complete recovery of ocular dominance to the originally deprived eye. This shift is accompanied by an increase in visual acuity and dendritic spine density (28). Although the underlying mechanisms by which PNNs halt plasticity remain elusive, a possible explanation for the protective action of PNNs is that they change the dynamics of local

GABAergic inhibition. PNNs form primarily around parvalbumin (PV)-positive GABAergic interneurons, which are involved in the onset of critical period in the visual cortex. Recent studies further showed that the expression of PNNs is regulated by the cellular transfer of the homeodomain transcription factor Otx2 from the retina and Choroid-plexus to the visual cortex. This homeoprotein signals PV maturation in GABAergic interneurons and contributes to both the opening and closure of the critical period (29–32) (**Figure 1A**).

In the fear system, the PNNs in the amygdala recently have been shown to play a central role in modifying the plasticity of fear memories and may contribute to protection against fear memory lability during acquisition. The number of CSPG containing PNNs increases in the murine amygdala between days 16 and 23 – the time coinciding with preadolescence and the developmentally related functional switch from fear erasure to less effective fear extinction (10) (**Figure 1B**). A recent study removing amygdala PNNs with chondroitinase-ABC supports the role of PNNs as a

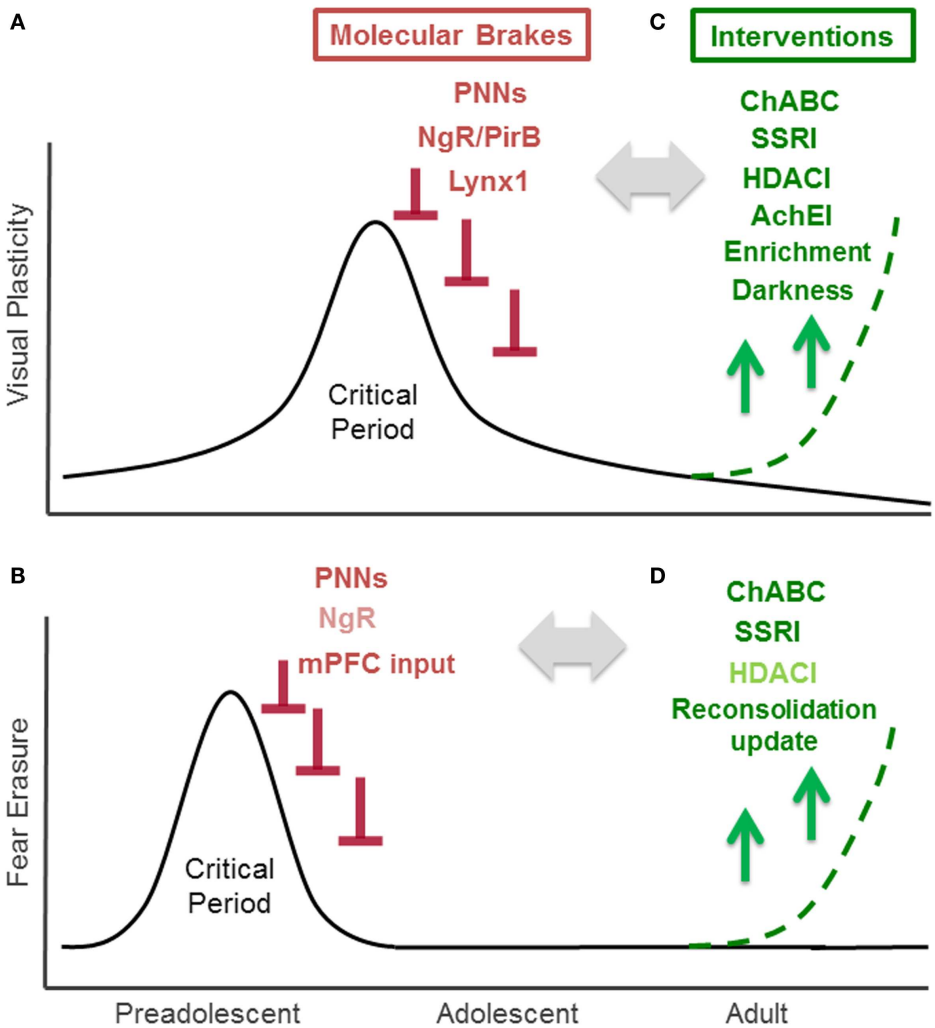

FIGURE 1 | Critical Period for experience-dependent plasticity in visual and fear system. **(A)** Critical period of visual cortex plasticity: visual cortex development is the preeminent model for the study of critical period plasticity and its regulators. Visually depriving one eye by obstructing it early in life yields a life-long loss of visual acuity (amblyopia). Studies of mouse visual cortex development, which has a well-defined 2 weeks critical period that peaks at 1 month after birth, have identified several endogenous "molecular brakes" (colored in red) that close the critical period. These include perineuronal nets (PNNs), myelin-related Nogo receptor (NgR) and PirB, and a nicotinic brake Lynx1. **(B)** Critical period of fear erasure: a critical period for the fear system near adolescence is emerging through current research. In rodents, extinction can permanently erase fear memory during a preadolescent critical period around 17 days after birth, however, extinction at 24 days or later fails to bar fear memories from re-emerging. Increased PNNs in the amygdala and maturation of input from medial prefrontal cortex (mPFC) to amygdala contribute to the closure of this critical period. Nogo receptor (NgR: colored in light red) also limits fear extinction, but its contribution on fear erasure is not tested. **(C)** Interventions for enhancing visual cortex plasticity in the adult: counteracting molecular brakes through pharmacological (ChABC, SSRI, HDACI, AChEI: colored in green) and behavioral (environmental enrichment, dark exposure) approaches is a promising therapeutic strategic for recovery from amblyopia. **(D)** Interventions for fear erasure in the adult: juvenile-like plasticity can be reintroduced in adulthood through pharmacological treatment (ChABC, SSRI treatment) or reconsolidation update. HDACI administration (colored in light green) enhances extinction, but its effect on fear erasure has not been examined yet.

plasticity brake that actively protects fear memories from erasure (10). When adult mice are injected with chondroitinase-ABC prior to fear conditioning and followed by extinction training 2–3 days later, fear extinction proceeds at a rate similar to that observed in juvenile mice, suggesting a greater response to extinction training (**Figure 1B**). Interestingly, chondroitinase-ABC was only effective when injected before fear conditioning; injection prior to extinction but after fear training was ineffective. Thus, the CPSG aggregation is likely involved in the initial fear memory encoding, and plays a role in maintaining the extinction-resistance of the fear memory rather than regulating the processes which occur during extinction. When the fear response was tested several weeks after the extinction training, the mice did not show a fear response to either the conditioned stimulus or the context of fear training, while control mice showed substantial renewal of the fear response (10). Thus formation of PNNs in fear systems may alter the function of local inhibitory circuits to promote the formation of extinction-resistant memory trace during fear conditioning, and degradation of these nets may allow for more inhibitory connectivity to protect against fear memory lability. In a post-mortem case control study, schizophrenic patients exhibited reduced numbers of PNNs in the lateral amygdala nuclei and entorhinal cortex (33). Further, oxidative stress, frequently observed in peripheral tissues and brains of schizophrenia patients, has been shown to delay the formation of PNNs (34, 35). The disruption of PNNs may play a central role in disease pathogenesis. It is currently difficult to discern whether the PNN is exerting an effect on fear acquisition or extinction because the downstream effectors of PNN disruption have not yet been identified. To better understand the role of the PNN in fear systems, further studies are needed to examine how PNNs are controlled and how they exert their effects.

MYELIN-RELATED NOGO RECEPTOR SIGNALING

In the visual cortex, experience-dependent plasticity is limited by myelin-related Nogo receptor signaling (23). The end of the visual critical period coincides with the maturation of intracortical myelin, which contains myelin-related inhibitory proteins such as NogoA, MAG, and OMgp, all of which bind to the neuronal Nogo receptor (36) (**Figure 1A**). Nogo receptor knock-out mice maintain normal levels of plasticity during the critical period, however the plasticity is maintained beyond critical period for up to 120 days postnatally (23). Additionally, PirB, a paired receptor with high affinity for Nogo, is also found to restrict ocular dominance plasticity in the visual cortex (24) (**Figure 1A**). The Nogo receptor was recently shown to be also involved in determining the rate of synaptic turnover in the adult cerebral cortex, as knock-out mice have increased levels of synaptic turnover (37). These myelin-related brakes may also regulate structural plasticity at the level of dendritic spine.

In a recent study, Nogo receptor knock-out mice were reported to show more pronounced fear extinction compared to wild type mice (37) (**Figure 1B**). At the dendritic spine level, fear extinction 3–4 days after fear conditioning involves spine growth on the same dendritic branches within 2 μm from the spines that were eliminated during conditioning in the cortex (38). Removal of a Nogo receptor brake may return high synaptic turnover which can result in stronger fear extinction learning (37). Whether or not Nogo receptor signaling contributes to fear erasure mechanisms in a fashion similar to PNNs is question that remains to be examined.

LYNX FAMILY

While structural brakes can limit plasticity by altering local connectivity, functional brakes can also halt plasticity by altering the neurotransmission between connections that have been formed to facilitate plasticity. In the visual cortex, a newly discovered class of proteins, the Lynx family, has been recently identified as a class of functional brakes (25). Lynx1, an endogenous prototoxin similar to α-bungarotoxin in snake venom, acts by binding to the nicotinic acetylcholine receptor (nAChR) and limiting its activation (39). Increases in Lynx1 expression coincide with closure of the critical period in the adult mouse visual cortex. Further, removal of this molecular brake during adulthood re-induces a plastic state by acutely resetting local excitatory-inhibitory circuit balance. Lynx1 expression in adults suppresses functional plasticity into a latent state, as removal of this brake allows the critical period remains open until nAChR signaling is actively blocked (25) (**Figure 1A**). The adult Lynx1 knock-out mice that received amblyopic long-term visual deprivation during critical period showed spontaneously recovery of visual acuity to normal levels simply by reopening the closed eye (25). While a permissive role for ACh has long been appreciated during the critical period (40), it has remained a mystery why visual cortex plasticity is severely restricted in adulthood even in the presence of massive cholinergic innervation from the basal forebrain. Lynx1 provides a molecular basis for maintaining stability in the presence of ACh.

The Lynx family may also have developmental roles in fear system. Lynx1 and closed related Lynx2 are both expressed in the amygdala and change its expression levels across development in both rodent and human (41). Both Lynx1 and Lynx2 knock-out mice express an amplified response to cue fear conditioning, but demonstrate normal contextual fear conditioning in adulthood (42, 43). As the juvenile cue response is normally stronger than that of the adult (44), Lynx family proteins may dampen cue-conditioned fear learning from adolescence to adulthood. Considering its age-related changes in expression, the Lynx family may also have a role on extinction and critical period of fear memory erasure. Indeed, nicotine administration during extinction training over the course of 6 days after fear conditioning is reported to enhance extinction (45), however, the direct role of Lynx family proteins in fear memory erasure remain to be tested.

THERAPEUTIC STRATEGIES BASED ON CRITICAL PERIOD MECHANISMS

The ability to remove brakes on critical periods provides the opportunity to reopen windows of plasticity in order to remodel, or re-develop, the adult brain by re-introducing juvenile-like plasticity. Small molecules targeting these brakes carry potential clinical relevance for both neurological and psychiatric disease, including PTSD. It is well-established that administering D-cycloserine (DCS), a partial NMDA agonist, facilitates extinction, and prevents the recovery of fear memories in rats, mice, and humans both before and after extinction training (46–49). Here we discuss the possibility of additionally using three well-established drug

classes administered in humans – selective serotonergic reuptake inhibitors (SSRIs), acetylcholinesterase inhibitors (AChEIs), and histone deacetylase (HDAC) inhibitors – to target both structural and functional plasticity brakes based on data from both animal and human studies (**Figure 1C**). Finally, we also consider behavioral interventions such as reconsolidation update, which may mimic or trigger similar effects.

SEROTONERGIC REUPTAKE INHIBITORS

In animal studies, chronic SSRI treatment reintroduces juvenile-like plasticity to the adult visual cortex. Administration of fluoxetine, the first-line antidepressant SSRI, has been demonstrated to restore critical period plasticity in the adult rat brain (50). Chronic fluoxetine treatment in adult rats not only induced visual cortex plasticity after monocular deprivation, but also improved visual acuity in amblyopic animals (**Figure 1C**). Specifically, rats were treated with fluoxetine for 4 weeks underwent monocular deprivation on day 21 of treatment. Increased BDNF levels in the visual cortex were accompanied by reduced GABAergic inhibition, likely restoring excitatory-inhibitory balance by reopening visual critical period plasticity.

Plasticity reactivation by chronic SSRI treatment has recently been examined in the fear system. Strikingly, fear erasure is facilitated in adult mice by SSRI treatment and resembles fear erasure in non-treated juvenile mice (**Figure 1D**) (51). Chronic fluoxetine treatment for 3 weeks prior to and throughout the duration of fear conditioning does not influence the encoding of a conditioned fear response, but rather causes faster extinction and permanent erasure compared to mice that undergo extinction training without prior SSRI treatment. This effect is mediated through BDNF pathways: BDNF expression was exaggerated in the basolateral amygdala by SSRI treatment, and the effect of SSRI on fear erasure was absent in mice heterozygous for BDNF allele. Interestingly, SSRI treatment starting after the fear acquisition was sufficient to induce faster extinction and permanent erasure, a salient difference compared to fear erasure induced by PNN disruption, which was only effective when treated before and not after fear conditioning. Fluoxetine treated mice had similar number of PNN-positive neurons compared to control but had a reduced percentage of PNN expressing parvalbumin interneurons in the basolateral amygdala. Together, these data suggest that fluoxetine treatment selectively shifts parvalbumin interneurons toward an immature state, inducing critical period-like plasticity in local inhibitory neurons in the basolateral amygdala. The effects of chronic fluoxetine treatment (4–5 weeks) were also seen in CA1 of the hippocampus, where SSRIs have been shown to return mature granule cells to an electrophysiologically immature state, with reduced synaptic facilitation in the mossy fibers (52). Loss of hippocampal synaptic proteins has been associated with a PTSD-like syndrome in mice, and is counteracted with SSRI treatment for 4 weeks of chronic fluoxetine treatment prior to fear conditioning (53).

ACETYLCHOLINESTERASE INHIBITORS

Another class of pharmaceutical agents shown to induce recovery of visual function in the adulthood is the acetylcholinesterase inhibitor (AchEI). In the visual system, AchEI injection can restore vision in adult wild type mice with amblyopia, a disorder in which the eye, though structurally normal, has impaired vision due to poor functional connectivity to the visual cortex (25) (**Figure 1C**). By increasing cholinergic tone, AChEI may counteract the nicotinic functional brake Lynx1 on critical period plasticity.

AchEI treatment has not been directly examined in the context of fear extinction and memory liability, but nicotine dosing immediately before training has been reported to enhance extinction training (45). As AChEI can rapidly activate BDNF receptor TrkB in hippocampus, AChEI may have similar effect on fear memory liability to SSRI (54). However, further studies are clearly needed to better elucidate the effects that AchEI have on fear system plasticity.

HDAC INHIBITORS

In the visual system, experience-dependent modifications of histone acetylation are developmentally down-regulated, implicating epigenetic mechanisms in the regulation of critical period plasticity (55). Administration of the HDAC inhibitor trichostatin A in adult mice reactivates visual cortex plasticity (55) and chronic administration of two separate HDAC inhibitors, valproic acid and sodium butyrate, to amblyopic adult mice undergoing long-term monocular deprivation induces recovery of visual acuity following reverse lid-suturing (56) (**Figure 1C**). Although the effect of HDAC inhibitors is intriguing, the changes in genetic expression profiles that HDAC inhibitors produce, and subsequent downstream effects of visual cortical plasticity, are still unknown. One possibility is that HDAC inhibitors are regulating gene expression of molecular brakes, such as myelin-related molecules, as histone modifications are involved in oligodendrocyte precursor cell differentiation during development. Some potential gene targets include transcription factors required for myelination, such as SOX10 and Krox-20. Administration of an HDAC inhibitor during myelination onset, which coincides with the fall of the visual cortex critical period, prevents oligodendrocyte precursor cell maturation (57–59). However, further analyses are required to unravel the effectors of the epigenetic treatment on visual plasticity and to confirm that effects are specific enough to plasticity brakes to be of clinical benefit.

In the fear system, systemic, as well as direct, application of HDAC inhibitors into the hippocampus or medial prefrontal cortex (mPFC) prior to fear conditioning and prior to extinction enhances extinction learning (60–64) (**Figure 1D**). Oral administration of an HDAC inhibitor enhances extinction learning in response to weak extinction protocols that are ineffective when administered on their own, and direct application of HDAC inhibitors to the hippocampus and mPFC increases c-fos expression. Future studies are necessary to determine whether HDAC inhibitors also promote permanent erasure of fear memory. Research comparing adult histone acetylation activity to juvenile activity will also inform whether acetylation has developmental specificity and mechanistic contributions to the critical period for fear lability.

BEHAVIORAL INTERVENTIONS

Visual system

Environmental enrichment (65) and dark exposure (66) have been reported as effective behavioral interventions for recovery from

amblyopia in adult rats (**Figure 1C**). These interventions may reset excitatory – inhibitory balance, thus re-introducing juvenile-like plasticity in the adult brain (8). The effects of behavioral interventions on recovery from adult amblyopia have also been examined in humans. Perceptual learning, involving extensive practice on a challenging discrimination between simple visual stimuli (67–69), and action videogames, which requires the allocation of spatial attention, detection, and localization of low contrast, fast moving targets, emerge as tools for visual acuity improvements in adult amblyopia (70, 71). These interventions may induce plasticity by either lifting molecular brakes through invasive interventions or by exploiting endogenous permissive factors such as neuromodulators (21).

Fear system

A newly proposed behavioral intervention for treating PTSD, "reconsolidation update," involves targeting traumatic memories as an individual reconsolidates it because the memory is rendered labile after it has been recalled (**Figure 1D**). Extinguishing a fear response during the window of reconsolidation prevents the reinstatement of a fear response to a stimulus at a later time point in both humans and in rodents (72–74). It is notable that the mechanisms of this process are localized to the amygdala and exclude the mPFC (75, 76). The mPFC is thought to be involved in reactivation of emotional states associated with past experiences, which may account for the lower rate of PTSD among war veterans with selective mPFC damage (77). The mPFC is also uninvolved in juvenile fear systems (**Figure 1B**) (78). Extinction during reconsolidation involves the amygdala in both juvenile mice and humans, and the mPFC is uninvolved in adolescent rat extinction (15, 79). During reconsolidation, events in the amygdala may open a temporal window of experience-dependent plasticity that allows a fear memory to be degraded by new experiences. However, it should be also noted that facilitation of extinction may also happen in addition to reconsolidation update (80). The molecular mechanisms of fear erasure by reconsolidation update are only now beginning to be explored. Recent work suggests that phosphorylation of the AMPA receptor subunit GluA1 regulates this process (72). There is also increasing evidence that reconsolidation is accompanied by epigenetic changes, pointing to a potential effect of epigenetic regulation on the cellular alterations underlying experience-dependent plasticity (81–85). Whether or not reconsolidation update limits the expression of molecular brakes remains open to future investigation. Combining reconsolidation update and pharmacological interventions may be a fruitful future direction.

CONCLUSION

In this review, we considered the potential contributions of "molecular brakes" identified in visual system development, the major model of critical period plasticity, to the development of fear system connections. Striking similarities between the molecular mechanisms underlying the development of these two brain regions, as well as therapeutic approaches to their dysfunction, indicate that new mechanisms identified in the visual critical period can provide both novel insights and a conceptual framework for exploring novel therapeutic approaches to aberrant fear responses in PTSD patients. Future studies examining the contributions of molecular brakes, such as myelin-related nogo receptor signaling and the Lynx family, as well as their epigenetic regulators in the context of the fear system development, may shed light on new targets for therapeutic intervention. The interactions between multiple brakes have yet to be connected – not only those mentioned in this review, but also well-established age-related mechanisms, such as hippocampal neurogenesis. Future research manipulating critical period mechanisms for clinical use will require further elucidation of systems in which these molecular brakes are involved. The mechanism of re-closure after the therapeutic reopening the critical period is another important area of investigation. Finally, better comprehension of the scope of these regulators to realize both their therapeutic potential as well as the undesired consequences of increased brain plasticity is needed.

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Exploring epigenetic regulation of fear memory and biomarkers associated with post-traumatic stress disorder

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This review examines recent work on epigenetic mechanisms underlying animal models of fear learning as well as its translational implications in disorders of fear regulation, such as Post-traumatic Stress Disorder (PTSD). Specifically, we will examine work outlining roles of differential histone acetylation and DNA-methylation associated with consolidation, reconsolidation, and extinction in Pavlovian fear paradigms. We then focus on the numerous studies examining the epigenetic modifications of the Brain-derived neurotrophin factor (*BDNF)* pathway and the extension of these findings from animal models to recent work in human clinical populations. We will also review recently published data on FKBP5 regulation of glucocorticoid receptor function, and how this is modulated in animal models of PTSD and in human clinical populations via epigenetic mechanisms. As glucocorticoid regulation of memory consolidation is well established in fear models, we examine how these recent data contribute to our broader understanding of fear memory formation. The combined recent progress in epigenetic modulation of memory with the advances in fear neurobiology suggest that this area may be critical to progress in our understanding of fear-related disorders with implications for new approaches to treatment and prevention.

Keywords: amygdala, fear memory, consolidation, reconsolidation, extinction, biomarkers, PTSD

INTRODUCTION

Pavlovian fear conditioning has become a useful tool for the identification of the cellular and molecular mechanisms necessary for the formation of fear memories (Levenson and Sweatt, 2005; Monsey et al., 2011; Mahan and Ressler, 2012; Zovkic and Sweatt, 2013; Zovkic et al., 2013). Moreover, Pavlovian fear conditioning as a model of traumatic memory formation has aided in the identification of potential intervention strategies, including extinction and reconsolidation-based memory interventions for the alleviation of traumatic fear memories (Andero and Ressler, 2012; Steckler and Risbrough, 2012). Recent work examining the cellular and molecular mechanisms necessary for the formation of Pavlovian fear memories has highlighted the significance of epigenetic mediation of traditional genomic targets already known to be critical for the formation of fear memories in animal models of post-traumatic stress disorder (PTSD). This review will address the recent progress that has been made in uncovering the epigenetic mechanisms necessary for auditory fear memory consolidation, reconsolidation, and extinction. We then address the translation of these animal findings into more recent work examining the correlation of epigenetic modifications at specific gene promoters associated with clinical PTSD.

EPIGENETIC MECHANISMS REQUIRED FOR AUDITORY FEAR MEMORY CONSOLIDATION

Decades of work have established that auditory fear memory consolidation requires genomic signaling cascades to mediate the transcriptional and translational processes which ultimately underlie fear memory formation (Johansen et al., 2011). Specifically, auditory fear conditioning results in the activation of ERK/MAPK within lateral amygdala (LA) neurons (Schafe et al., 2000). ERK/MAPK in turn translocates to the nucleus where it phosphorylates the transcription factor CREB to mediate downstream transcriptional activation (Josselyn et al., 2001; Ressler et al., 2002; Ploski et al., 2010). While much progress has been made examining these traditional genomic signaling mechanisms, it has become increasingly evident that additional mechanisms likely also influence and regulate the transcriptional access necessary for synaptic plasticity and memory formation (Levenson and Sweatt, 2005, 2006; Barrett and Wood, 2008; Jiang et al., 2008; Zovkic and Sweatt, 2013; Zovkic et al., 2013). Thus in recent years attention has turned to examining how "epigenetic" mechanisms may regulate transcriptional access to genes which are critical for memory formation.

Epigenetics involves the study of changes in gene expression which occur independent of alterations to the underlying DNA sequence. Two epigenetic mechanisms in particular have been examined in memory formation and synaptic plasticity: post-translational modifications to chromatin structure and DNA methylation (Levenson and Sweatt, 2005). Within the nucleus, DNA is tightly condensed into chromatin consisting of eight histones: two copies each of H2A, H2B, H3, and H4 as well as the linker histone H1. Each histone possesses an N-terminus tail capable of undergoing multiple modifications,

including acetylation, phosphorylation, and methylation (Levenson and Sweatt, 2005). While a few studies have identified the regulation of histone methylation and phosphorylation accompanying fear conditioning in contextual fear paradigms (Chwang et al., 2006; Gupta et al., 2010; Gupta-Agarwal et al., 2012), histone acetylation has been more commonly studied within the context of learning and memory (Graff and Tsai, 2013). Positively charged lysine residues on N-terminus histone tails restrict transcriptional access and the acetylation of these residues via histone acetyltransferases (HATs) neutralizes the positive charges on the histone tails to relax chromatin structure and promote accessibility for transcription factor binding (Varga-Weisz and Becker, 1998; Yang and Seto, 2007). Conversely, histone acetylation is negatively regulated by histone deacetylases (HDACs) which remove acetyl groups from lysine residues and thus condense chromatin structure and interfere with transcriptional access (Varga-Weisz and Becker, 1998; Yang and Seto, 2007) (**Figure 1**).

Whereas histone acetylation has been widely associated with transcriptional activation, DNA methylation has traditionally been considered a static process associated with transcriptional repression (Miranda and Jones, 2007). DNA methylation is the process whereby methyl groups are added to cytosine residues within the DNA sequence and the addition of these methyl groups has been shown to inhibit transcriptional access to DNA, a process which is catalyzed by DNA methyltransferases (DNMTs) (Miranda and Jones, 2007). While the existence of dynamic DNMT activity within the mature central nervous system (CNS) has been documented, preliminary evidence has emerged to reveal a role for Gadd45b as a regulator of active DNA demethylation within the CNS (Leach et al., 2012; Sultan et al., 2012) (**Figure 1**).

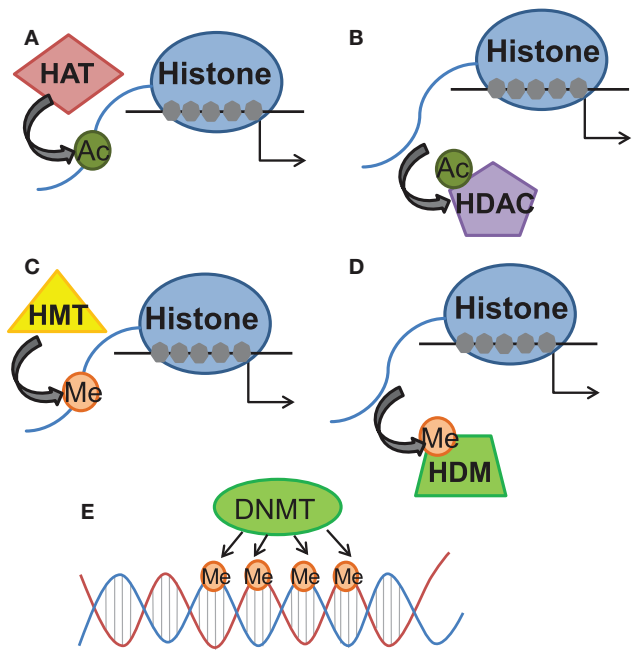

FIGURE 1 | Schematic diagram of histone and DNA-methylation processes of epigenetic regulation of gene expression. The schematic diagram demonstrates the primary known functions of the different enzymes referred to within the review. **(A)** Histone acetyltransferases (HAT) add acetyl groups to histones, generally associated with relaxing wound DNA. **(B)** Histone deacetylases (HDAC) remove those acetyl groups. **(C)** Histone methyltransferases (HMT) add methyl groups to histones, generally associated with tightening wound DNA. **(D)** Histone demethylases (HDM) remove those methyl groups. **(E)** DNA methyltransferases (DNMT) add methyl groups to DNA, sometimes associated with DNA silencing.

Early evidence of a role for chromatin modifications accompanying learning in mammalian models of learning and memory was revealed by a study employing a contextual fear conditioning paradigm known to regulate the ERK/MAPK–CREB genomic signaling pathway. This study from David Sweatt's group revealed robust regulation of histone H3 acetylation, but not H4, 60 min following contextual fear conditioning in area CA1 of the hippocampus (Levenson et al., 2004), a pattern of findings which has been well-replicated (Lubin and Sweatt, 2007; Miller et al., 2008). Further, increasing histone acetylation via HDAC inhibition has been well documented to enhance memory consolidation in a variety of hippocampal-dependent learning paradigms, including object recognition and contextual fear conditioning (Levenson et al., 2004; Stefanko et al., 2009; but see Sintoni et al., 2013).

While regulation of histone H3 and H4 acetylation have been most commonly examined and acetyl-H3 regulation is most widely reported, evidence has emerged implicating the regulation of histone H2B acetylation accompanying contextual fear and spatial learning in the hippocampus suggesting not only that there may be differential regulation of specific histones with varying types of memory formation but also that a closer look at the regulation of other histones with learning and memory is warranted (Bousiges et al., 2013). Further, it is worth noting that learning-related alterations in histone acetylation have not been ubiquitously observed. One such study employing an invertebrate model of context-signal memory, whereby presentation of a danger stimulus elicits an escape response, demonstrated that while HDAC inhibition was capable of enhancing the escape response and accompanying levels of histone acetylation with weak training procedures, only strong training was capable of resulting in robust regulation of histone acetylation (Federman et al., 2009). Further, another study employing a food aversion paradigm in the mollusk revealed asymmetric regulation of histone acetylation accompanying training (Danilova et al., 2010). These studies suggest that there may be important training-related gradients which determine the extent to which histone acetylation is regulated by learning and that there may be asymmetric differences in the engagement of histone acetylation accompanying training. However, despite these findings from invertebrate learning and memory models, the training parameters necessary to induce alterations in histone acetylation events or the existence of asymmetric regulation within mammalian models has yet to be determined.

Further examination of the regulation of histone acetylation in mammalian models has demonstrated that the observed training-related regulation of histone H3 acetylation is downstream of ERK/MAPK signaling, as the MEK inhibitor U0126 was found to impair the acetylation of H3 (Levenson et al., 2004). Inhibition of ERK/MAPK signaling has also been found to impair histone acetylation regulation in an invertebrate model of food aversion learning (Danilova et al., 2010) and in the consolidation of auditory fear memories (Monsey et al., 2011). These findings

implicate the interplay of traditional genomic signaling cascades and epigenetic mechanisms. They also suggest the existence of a level of epigenetic regulation of transcriptional processes necessary for memory formation, which has only become more widely appreciated within the last decade.

EPIGENETIC UNDERPINNINGS OF FEAR MEMORY

HISTONE ACETYLATION MEDIATES AUDITORY FEAR MEMORY CONSOLIDATION

Early work examining the necessity of epigenetic mechanisms in the regulation of memory formation has largely employed hippocampal-dependent memory paradigms (Levenson et al., 2004; Miller and Sweatt, 2007; Lubin et al., 2008; Miller et al., 2008; Stefanko et al., 2009). More recent work has begun to examine the role of epigenetic mechanisms in amygdala-dependent memory processes. In agreement with the evidence for regulation of histone acetylation with contextual fear conditioning, a recent series of studies has demonstrated that auditory fear conditioning also results in an increase in histone H3 but not H4 acetylation in the LA (Monsey et al., 2011). Further, this study demonstrated that increasing histone acetylation via HDAC inhibition in the LA resulted in enhanced auditory fear memory consolidation, i.e., freezing during long-term memory was enhanced whereas short-term memory was not affected (**Figure 2**).

Despite the documented increase in histone H3 acetylation with auditory fear conditioning and facilitation of fear memory consolidation via HDAC inhibition, these findings did not directly address the necessity of histone acetylation in fear memory consolidation. To date, while many proteins have been identified which possess HAT activity, three in particular have been widely examined within the field of learning and memory: E1a-associated protein (p300), CREB binding protein (CBP), and p300-CBP-associated protein (PCAF) (Barrett and Wood, 2008). Much of the work examining the role of these HATs in memory processes have employed transgenic mouse models of varying degrees from brain-region specific to full knockout of HAT proteins (Oike et al., 1999; Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2005, 2006; Oliveira et al., 2007, 2011; Valor et al., 2011). From this wide collection of studies only two have revealed deficits in amygdala-dependent auditory fear memory (Oike et al., 1999; Alarcon et al., 2004), while many

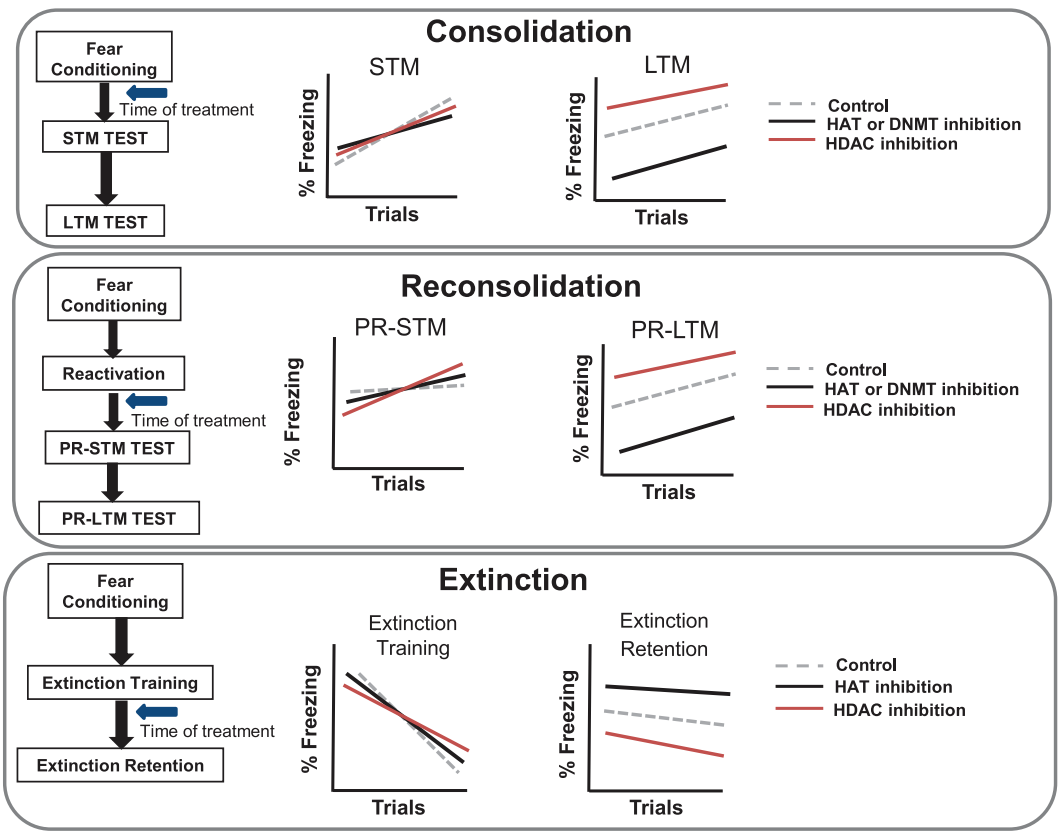

FIGURE 2 | Schematic diagram of different phases of fear learning, and predicted effects of inhibiting histone acetyltransferase, histone deacetylase, or DNA methyltransferase. The schematic diagram illustrates the predicted outcome of pharmacological inhibition of histone and DNA modifying enzymes on the primary aspects of fear memory. (Top) When treated with inhibitors during the consolidation phase of fear conditioning, short-term memory (STM) tests are not generally effected, but long-term memory (LTM) expression is impaired with HAT and DNMT inhibition or increased with HDAC inhibition. (Middle) When inhibitors are given after a brief memory reactivation, they may affect memory reconsolidation processes. In this case, there are no predicted effects on short-term memory post-reactivation (PR-STM); however, PR-LTM is impaired with HAT and DNMT inhibition or increased with HDAC inhibition. (Bottom) When inhibitors are given following extinction training, HAT inhibition is predicted to impair extinction retention while HDAC inhibition is predicted to enhance extinction retention.

report deficits in the hippocampal-dependent tasks of contextual fear and the novel object recognition paradigm (Korzus et al., 2004; Wood et al., 2005, 2006; Oliveira et al., 2007, 2011; Valor et al., 2011).

Recently, pharmacological inhibitors of HAT activity have become commercially available and these agents have been employed in specific brain regions to examine more targeted inhibition of HAT proteins in conjunction with learning and memory paradigms, independent of the potential developmental confounds with transgenic models (Marek et al., 2011; Wei et al., 2012; Zhao et al., 2012). Inhibition of HAT activity using either garcinol or c646 administration accompanying auditory fear conditioning has been found to inhibit training-related changes in histone acetylation within the LA and coordinately inhibit auditory fear memory consolidation, in a temporally graded manner (Maddox et al., 2013a,b). Further, these studies demonstrated that inhibition of HAT activity shortly following auditory fear conditioning also impaired learning-related enhancements in auditory-evoked field potentials (AEFPs) within the LA, a commonly studied neurophysiological correlate of auditory fear memory consolidation (Quirk et al., 1995; Rogan et al., 1997). It is worth considering that given the large sequence homology between CBP and p300, and given the ability of CBP/p300 to structurally associate with PCAF to regulate histone acetylation (Schiltz et al., 1999), making claims about the necessity of one HAT over another using these pharmacological inhibitors is not warranted. While these data do reveal a critical role for HAT activity in the consolidation of auditory fear memories, more attention is needed to examine the specific contributions each of these HATs may make either independently or in combination with one another to promote learning and memory.

A recent study examining the role of PCAF in auditory fear memory extinction suggests that PCAF in the infra-limbic prefrontal cortex (ILPFC) has a selective role in the consolidation of auditory fear memory extinction (Wei et al., 2012). However it is worth noting that in an experiment from this study, the PCAF inhibitor H3-CoA-20-Tat was infused into the prelimbic PFC (PLPFC) immediately following auditory fear conditioning and was found to enhance consolidation of auditory fear memory. A role for the PLPFC in expression and consolidation of fear memories has been established (Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007; Choi et al., 2010). Further, a role for brain-derived neurotrophin factor (BDNF) in the PLPFC has been identified as a key mediator of fear memory consolidation (Choi et al., 2010), and as recent work has shown that HDAC administration can enhance levels of BDNF mRNA (Zeng et al., 2011) (*and see below)*, the mechanism though which inhibition of HAT activity in the PLPFC results in enhanced fear memory consolidation remains unclear.

DNA METHYLATION MEDIATES AUDITORY FEAR MEMORY CONSOLIDATION

While traditionally considered a stable and enduring transcriptional constraint, studies examining the role of DNA methylation in learning and memory have revealed dynamic regulation of DNA methylation (Miller and Sweatt, 2007; Lubin et al., 2008; Feng et al., 2010; Han et al., 2010). As DNMTs serve to add methyl groups to CpG islands on DNA sequences to silence gene expression, traditional logic suggests that their inhibition would enhance memory formation via transcriptional activation. Conversely, studies have shown that inhibition of DNMT activity interferes with memory consolidation processes in a variety of hippocampal-dependent learning paradigms, including contextual fear, cocaine-induced place preference, and Morris water maze learning (Miller and Sweatt, 2007; Lubin et al., 2008; Feng et al., 2010; Han et al., 2010). In agreement with these findings, auditory fear conditioning has recently been associated with an increase in DNMT3a protein within the LA (Monsey et al., 2011). Inhibition of DNMT activity within the LA at or near the time of training results in impaired fear memory consolidation, suggesting that DNMT activity is critical for amygdala-dependent fear memory consolidation as it is in the hippocampal contextual fear memory system (Monsey et al., 2011). In support of these findings, experiments employing slice electrophysiology, revealed that inhibition of DNMT activity impairs long-term potentiation (LTP), a well established model of synaptic plasticity, from both thalamic and cortical input pathways to the amygdala. Thus, this pattern of findings clearly implicates DNMT activity in the establishment of synaptic plasticity and ultimately auditory fear memory consolidation.

While these early studies implicate a role for DNMT activity in memory consolidation processes, only recently has evidence emerged to suggest the existence of a DNA demethylase within the CNS. Gadd45b has been proposed to be a regulator of active DNA demethylation within the adult CNS (Leach et al., 2012; Sultan et al., 2012). Both of these studies employ a genetic knockout model of Gadd45b yet draw contrasting conclusions regarding the role of Gadd45b in memory processes. While one study revealed enhancements in contextual fear memory consolidation with mild and moderate training parameters and implicates a role of Gadd45b as a negative regulator of contextual fear memory formation (Sultan et al., 2012), the other study demonstrated impaired contextual fear memory consolidation and is in line with a permissive role for Gadd45b in memory formation (Leach et al., 2012). However, both studies failed to demonstrate an effect on the consolidation of amygdala-dependent auditory fear memory, suggesting that the identification of an active DNA demethylase in the auditory (or cued) fear memory system has yet to be determined. While the findings of enhanced memory formation accompanying Gadd45b knockout are in line with the findings demonstrating memory consolidation deficits associated with DNMT inhibition (Miller and Sweatt, 2007; Miller et al., 2008), there remains a lack of cohesion regarding the role of Gadd45b in contextual fear memory formation. These findings demonstrate a clear need for further investigation into the underlying mechanism through which Gadd45b may function as a regulator of memory formation.

Although multiple labs have now documented fear memory consolidation deficits accompanying DNMT activity inhibition in either the amygdala or the hippocampus, the findings are still somewhat counter-intuitive. However, a few studies have provided evidence for a possible mechanism underlying these memory deficits. A series of studies has revealed an interaction between DNA methylation and histone acetylation in the consolidation of contextual and auditory fear memories (Miller et al., 2008; Monsey et al., 2011). Both studies demonstrated that memory consolidation deficits accompanying DNMT inhibition were associated

with impaired training-related regulation of histone acetylation but that consolidation and histone acetylation deficits could be rescued with HDAC inhibition. Thus, these findings suggest that one method by which DNMT inhibition may result in memory consolidation deficits is via its inhibition of the critical training-related regulation of histone acetylation and suggest that there is a critical cooperation between these epigenetic mechanisms which is necessary for memory consolidation.

Another compelling hypothesis for the memory consolidation deficits associated with DNMT inhibition concerns the differential effect on DNA-methylation patterns across various subsets of genes. First, work has suggested that the methyl-binding protein, MeCP2, interacts with the transcription factor CREB1 to regulate the activation of CRE-mediated genes, suggesting that methylation and the binding of methyl-binding proteins (MBPs) at CRE sites is associated with transcriptional activation (Chahrour et al., 2008). In agreement with this finding, work from Farah Lubin's group (Gupta et al., 2010) demonstrated that contextual fear conditioning results in an increase in DNA methylation within the *zif268* promoter region and a corresponding increase in zif268 mRNA expression. Moreover this group demonstrated an increase in MeCP2 within the *zif268* promotor region associated with DNA methylation, suggesting that indeed methylation is required for the activation of CRE-mediated genes such as *zif268*. Thus these findings may suggest that inhibition of DNMT activity near the time of fear conditioning results in consolidation deficits via the inhibition of CRE-mediated genes which are critical for consolidation, such as *zif268* (Maddox et al., 2011), and suggest that DNA methylation can in some cases be associated with transcriptional activation.

An extension of this hypothesis concerns the possibility that DNMT inhibition may offset the balance of memory-promoting (e.g., the CRE-mediated IEG *zif268*) and memory-suppressive genes. Contextual fear conditioning has been associated with demethylation and a corresponding increase in *reelin* mRNA, a memory-promoting gene, in the hippocampus. Conversely, contextual fear learning leads to a hypermethylation of the memory-suppressive gene protein phosphatase (*PP1)* with a corresponding reduction in *PP1* mRNA (Miller and Sweatt, 2007). Further, DNMT inhibition was found to reverse these changes, such that the training-induced methylation of *PP1* was impaired and thus *PP1* mRNA was increased. These data suggest that one manner in which DNMT inhibition results in impaired memory consolidation is via the demethylation of memory-suppressing genes, such that their enhanced expression results in memory impairment. Further, it remains possible that the findings of DNMT inhibition's effects on (1) the reduction of training-related changes in histone acetylation, (2) ability to enhance the transcription of memory-suppressive genes, and (3) its impairment of memory-promoting genes, especially the induction of CRE-mediated IEGs, are not mutually exclusive events. This suggests that these effects should be considered in concert when further examining the mechanism through which DNMT inhibition impairs memory consolidation.

EPIGENETIC REGULATION OF AUDITORY FEAR MEMORY RECONSOLIDATION

Another growing field of study within the realm of epigenetic-mediation of fear memories is the examination of a role for epigenetic processes in the reconsolidation of auditory fear memories. Reconsolidation is the phenomenon whereby retrieval of a previously acquired memory results in the induction of a period of instability during which the memory may be updated, either strengthened or weakened, prior to being re-stabilized (Nader et al., 2000; Tronson and Taylor, 2007). An early study noted the existence of epigenetic mechanisms in contextual fear memory reconsolidation by revealing the retrieval-induced regulation of histone acetylation in area CA1 of the hippocampus via the NF-κB/IKK (Nuclear Factor Kappa-light-chain-enhancer of activated B cells/inhibitor of NF-κB kinase) pathway (Lubin and Sweatt, 2007). Within the last few years, a series of studies has further contributed to this early work by outlining a critical role for epigenetic mechanisms in auditory fear memory reconsolidation. As with initial auditory fear memory consolidation, retrieval of a previously acquired auditory fear memory was found to result in a retrieval-dependent increase in histone H3 acetylation, but not regulation of H4 acetylation in the LA (Maddox and Schafe, 2011). Moreover, HDAC inhibition accompanying auditory fear memory retrieval was found to enhance memory reconsolidation in a retrieval-dependent and temporally graded manner, suggesting that as with auditory fear memory consolidation, HDAC activity appears to negatively regulate fear memory reconsolidation within the LA (**Figure 2**). To further explore the role of histone acetylation in fear memory reconsolidation, more recent work has revealed that HAT activity is critical in mediating retrieval-related alterations in histone acetylation and that HAT inhibition impairs fear memory reconsolidation (Maddox et al., 2013a,b). Results from both studies have demonstrated that inhibition of HAT activity results in a long-lasting and robust reconsolidation deficit which is dependent on memory retrieval, insensitive to spontaneous recovery, reinstatement, and fear renewal in a novel context. Further, these studies demonstrated that inhibition of HAT activity accompanying fear memory retrieval was capable of reversing the underlying memory-associated changes in AEFPs, suggesting that this memory intervention strategy is effective at impairing fear memory reconsolidation at the level of behavior and at the level of synaptic plasticity, *in vivo*. Moreover, as garcinol is a naturally occurring HAT inhibitor, these data were the first to document the pre-clinical efficacy of a naturally occurring pharmacological strategy in conjunction with reconsolidation-based fear memory intervention. These findings highlight the potential for future identification of naturally occurring compounds to use in the treatment of fear and anxiety disorders (Maddox et al., 2013a).

Comparatively fewer studies have examined the role of DNA methylation in auditory fear memory reconsolidation processes; however in agreement with the findings of studies of auditory fear memory consolidation, intra-LA inhibition of DNMT activity at or near the time of auditory fear memory retrieval has been shown to impair the reconsolidation of memory (Maddox and Schafe, 2011). Moreover, inhibition of DNMT activity accompanying fear memory retrieval was found to result in a retrieval-dependent, temporally graded deficit which is insensitive to spontaneous recovery, reinstatement, and fear renewal. While these data demonstrate a critical role for DNMT activity in reconsolidation as one method to alleviate existing fear memories, a role for DNA methylation and DNMT activity has yet to be revealed

for the consolidation of fear extinction memory, the other widely explored method for alleviating existing fear memories.

EPIGENETIC REGULATION OF FEAR MEMORY EXTINCTION

Rodent models of fear memory extinction have proven quite useful in making suggestions about relevant pharmacological manipulations which may be implemented in cognitive-behavioral therapy for the treatment of phobias, anxiety disorders, and PTSD (Andero and Ressler, 2012). Extinction involves the repeated presentation of the fear-invoking conditioned stimulus (CS) in the absence of the unconditioned stimulus (US), and results in the formation a new memory which inhibits the expression of the existing fear memory (Myers and Davis, 2007). Given that HDAC inhibitors have been widely reported to enhance memory, recent work has turned toward examining the potential for HDAC inhibition to enhance extinction memory as it may have clinical implications. A few labs have now provided evidence for the benefit of HDAC inhibition on fear extinction learning by demonstrating that systemic HDAC inhibition is capable of facilitating extinction for auditory fear memory (Lattal et al., 2007; Bredy and Barad, 2008; Fujita et al., 2012; Itzhak et al., 2012) (**Figure 2**) and facilitating extinction of cocaine conditioned place preference (Malvaez et al., 2010).

However, there is some evidence to suggest that there may be limits on the effectiveness of HDAC inhibition in facilitating extinction. One such study demonstrated that intra-hippocampal or intra-ILPFC administration of the HDAC inhibitor sodium butyrate (NaB) was capable of enhancing contextual fear memory extinction, but only under conditions when extinction training was weak, i.e., NaB administration was not effective in facilitating extinction using more stringent extinction sessions (Stafford et al., 2012). The lack of a facilitation of extinction with HDAC inhibition in conjunction with stronger extinction parameters may suggest the presence of a ceiling effect. However, extinction facilitation accompanying HDAC inhibition has not always proven successful (Kilgore et al., 2010). Another study has suggested that while overexpression of HDAC1 in the hippocampus facilitates contextual fear extinction, inhibition of HDAC1 correspondingly results in impaired extinction (Bahari-Javan et al., 2012). As HDAC inhibition has been associated with enhanced extinction in a number of studies using pharmacological approaches such as NaB, Vorinostat, or TSA, HDAC inhibitors which inhibit multiple classes of HDACs, it remains unclear if different classes of HDACs make different contributions to fear memory extinction. The lack of a cohesive role for HDAC activity in fear memory extinction clearly suggests that this is an area of work which requires further investigation.

Another study has demonstrated that inhibition of PCAF activity within the ILPFC impairs the consolidation of extinction for an auditory fear (Wei et al., 2012), data which implicate a critical role for histone acetylation via HATs in fear memory extinction. However, it is worth noting that while many studies have demonstrated extinction enhancements accompanying HDAC inhibition, one study has shown that inhibition of the HAT activity of p300/CBP paradoxically enhances extinction memory (Marek et al., 2011). The existence of the same behavioral outcome (i.e., enhanced extinction via both inhibition and enhancement of histone acetylation) is somewhat perplexing and further research is warranted to uncover the mechanism underlying these findings. As this lab has provided evidence for a critical role for PCAF in the consolidation of auditory fear extinction (Wei et al., 2012) further investigation might reveal a unique relationship between these HATs and their role in the regulation fear memory extinction.

Intriguingly, a recent study examining the potential chromatin modifications in the ventral-medial PFC (vmPFC) occurring with extinction of conditioned place aversion (CPA) accompanying morphine withdrawal (Wang et al., 2012) demonstrated increased AcH3 and AcH4 following extinction. This study further demonstrated that intra-vmPFC administration of the partial NMDA-receptor agonist D-cycloserine (DCS) resulted not only in enhanced CPA extinction, but also facilitated extinction-related increases in AcH3 and AcH4. As there is already evidence for the efficacy of DCS in the treatment of fear memory in rodent models (Walker et al., 2002) and in phobias in a clinical population (Ressler et al., 2004), these data may suggest that one unappreciated mechanism through which DCS may be effective in promoting extinction-related plasticity is via enhancement of chromatin modifications which are downstream of NMDAR activation.

TRANSLATING ANIMAL MODELS TO THE CLINIC: EPIGENETIC MODIFICATIONS OF GENES ASSOCIATED WITH FEAR AND ANXIETY DISORDERS

Much work has revealed the regulation of global epigenetic alterations, especially histone acetylation and DNA methylation, accompanying both auditory fear conditioning and memory retrieval in the amygdala and accompanying contextual fear conditioning in the hippocampus. Additionally, some progress has been made in revealing the epigenetic regulation of specific genes associated with fear memory. Animal models examining the impact of early-life trauma, stress, and adversity as a risk factor for the future development of psychiatric disorders appear to have gained support from recent studies in human clinical populations. Moreover, it is the examination of epigenetic-modifications associated with specific genes which has recently been explored within the context of clinical PTSD and other psychiatric disorders. Given recent work examining epigenetic risk factors associated with the development of PTSD, we now review work employing animal models of epigenetic regulation of anxiety and fear associated genes which has been translated into human clinical populations, including epigenetic modifications of glucocorticoid receptor (GR) function including *FKBP5*, *Nr3c1*, and the *BDNF* pathways.

THE BDNF-TrKB PATHWAY AND ITS DOWNSTREAM EFFECTORS

Brain-derived neurotrophin factor is a neurotrophic factor which has been widely implicated in nervous system development, synaptic plasticity, and has been shown to be highly enriched in brain-regions associated with emotional learning including the amygdala, hippocampus, and PFC (Hofer et al., 1990). Importantly, support for a role for BDNF in emotional learning in animal models has been recently translated to PTSD in humans, as illuminated by the finding that individuals with the BDNF polymorphism Val66Met have impaired fear memory extinction (Soliman et al., 2010). While a role for the BDNF peptide and its receptor, TrkB,

have been well established in auditory fear conditioning (Rattiner et al., 2004; Choi et al., 2010; Andero et al., 2011), additional insight into the epigenetic mechanisms regulating the BDNF pathway and its role in PTSD have been investigated by a number of groups.

Brain-derived neurotrophin factor consists of nine 5′-non-coding exons, each possessing individual promoter regions and one 3′-coding exon (IX) which codes for the BDNF pre-protein amino acid sequence (Aid et al., 2007). The existence of these nine individual promoter regions within the BDNF gene sequence allows for the potential for unique BDNF transcripts with differential regulatory regions and has been proposed to ultimately underlie functional differences in BDNF transcription (e.g., Rattiner et al., 2004). Due to this potential for functional differences in BDNF accompanying differential regulation of individual BDNF exons, studies exploring the epigenetic regulation of BDNF have examined multiple BDNF exons. Using a contextual fear memory paradigm, it was determined that context-shock associations resulted in a selective decrease in DNA methylation within BDNF exon IV and a corresponding increase in BDNF exon IV mRNA in the hippocampus (Lubin et al., 2008). In addition, a study employing a PTSD model, examining the impact of prior-stress experience on subsequent contextual fear memory formation, determined that prior-stress experience resulted in enhanced freezing levels and correspondingly an increase in BDNF exon I and IV mRNA in the hippocampus (Takei et al., 2011). This study further revealed an increase in histone H3 and H4 acetylation within exon I and IV BDNF promoters, implicating a role for histone acetylation-mediated enhancement of BDNF mRNA accompanying stress-enhanced contextual fear memory formation. While this study is in agreement with the previous implication of epigenetic regulation of BDNF exon IV in contextual fear memory formation, the finding of significant regulation of BDNF exon I regulation with stress-enhanced contextual fear learning may suggest that stress allows for more extensive epigenetic modifications within the BDNF gene to promote fear memory formation. However, it is worth noting that a previous study from this group examining the epigenetic regulation of BDNF following a single session of immobilization stress in the hippocampus revealed a reduction in AcH3 association with BDNF exon I and IV promoters and a corresponding decrease in BDNF I and IV transcript mRNA 2 h following stress, but not 24 h (Fuchikami et al., 2009). As these findings suggest that previous stress can enhance histone acetylation-mediated BDNF transcription accompanying contextual fear conditioning 1-week later (Takei et al., 2011), the immediate post-stress reduction in BDNF transcription, and histone-modification seems at odds with this hypothesis. Thus, more work is necessary to examine how prior-stress experience can enhance histone acetylation-mediated BDNF transcription at this later time point, when stress experience itself does not appear to have a long-lasting (24 h later) effect on BDNF transcription in the hippocampus.

Although studies examining the regulation of histone acetylation-mediation of BDNF transcription have not revealed enduring changes in acetylation-mediated regulation of specific BDNF promoter transcripts, suggesting that chromatin modifications are transiently mediated following stress and/or fear conditioning, work examining DNA-methylation of BDNF promoters has revealed more enduring epigenetic regulation of BDNF. Using a psychosocial-stress PTSD model in rats, methylation of BDNF promoters was examined following a 1 month stress period (Roth et al., 2011). BDNF exon IV was found to have enhanced levels of CpG methylation in both the dentate gyrus and area CA1 of the hippocampus in the stress exposed group and a corresponding reduction in BDNF exon IV mRNA levels. However, this study failed to find stress-related alterations in BDNF DNA methylation in the amygdala and PFC. These data suggest that there is a robust regulation of DNA methylation at CpG islands within BDNF exon IV in the hippocampus which is a consequence of stress. It remains unclear why no changes were observed in the amygdala and PFC, both structures which have been shown to be affected by experience with stress (Rodrigues et al., 2009). However, the epigenetic-regulation of BDNF exon IV in the hippocampus is in agreement with the aforementioned findings and signifies that exon IV in particular undergoes robust epigenetic-modifications accompanying contextual fear memory formation and stress exposure. Thus this BDNF promoter/exon regulation is likely key in the development of a PTSD-like condition in these animal models.

Translational work employing a rodent model of early-life maltreatment and trauma has demonstrated long-lasting epigenetic-mediation of BDNF transcription (Roth et al., 2009). In this study, infant rats were exposed to a stressed, "abusive" mother for 30 min per day during the first post-natal week or were cross-fostered by a non-abusive mother for the same period of time. Upon adulthood it was determined that those rats who experienced maltreatment by the abusive mother had significant reductions in total BDNF mRNA (exon IX) in the hippocampus, compared to the cross-fostered non-maltreated adults, suggesting that there is a long-lasting effect of early-life maltreatment on BDNF regulation. Further, this study revealed an increase in BDNF exon IV and IX DNA methylation in the PFC of adult rats with a history of early post-natal maltreatment, suggesting that early-life stress is capable of resulting in long-lasting epigenetic changes at BDNF promoters which result in long-lasting BDNF transcription deficits.

These studies reveal a role for epigenetic-mediated regulation of BDNF transcription accompanying contextual fear memory formation, and demonstrate an enhancement of epigenetic-mediated BDNF transcription with prior-stress experience. To date only one study has examined the potential role for epigenetic-mediation of BDNF transcription accompanying fear memory extinction (Bredy et al., 2007). This study demonstrated that while auditory fear conditioning was associated with increased AcH3 at BDNF I and IV exon promoters in the PFC, extinction was associated with a decrease in AcH3 recruitment to the exon I promoter but increased AcH4 at BDNF exon IV promoter. Further, while exon IV mRNA was enhanced with fear conditioning, extinction was found to result in a more profound increase in exon IV mRNA and also to induce an increase in exon I mRNA. These data are the first to reveal differential regulation of BDNF transcripts in the PFC accompanying fear memory formation and extinction learning and suggest that there may be different epigenetic-mediation of BDNF transcription for different forms of memory, i.e., consolidation versus extinction.

While these early studies have laid a firm foundational role for epigenetic regulation of BDNF accompanying fear memory and/or

stress exposure in a variety of paradigms, recent work has identified epigenetic regulation of *homer1a*, a gene variant of *homer1* and a downstream transcriptional target of BDNF-TrkB signaling (Mahan et al., 2012). Using a fear conditioning paradigm which is capable of instating both auditory and contextual fear memory-related plasticity, this study revealed that fear conditioning was associated with an increase in histone H3 acetylation within the *homer1* promoter region within the hippocampus while a decrease in histone H3 methylation within the *homer1* promoter region was observed within the amygdala (**Figure 3**). Both of these changes in histone H3 modifications were associated with an increase in *homer1a* mRNA, suggesting that there is differential and brain-region specific epigenetic regulation of *homer1a* expression. As with the earlier suggestion of differential epigenetic regulation of BDNF transcription for fear memory formation and extinction memory in the PFC (Bredy et al., 2007) these data may suggest that contextual fear memories mediated by the hippocampus may require histone acetylation-mediated *homer1a* transcription, whereas auditory fear memories mediated by the amygdala may require a decrease in H3 methylation to induce critical regulation of *homer1a* with memory formation.

As noted earlier, while a variety of animal models of fear and anxiety disorders have demonstrated epigenetic regulation of BDNF, only a few studies have revealed the potential role for epigenetic regulation of BDNF in human clinical populations. For example, a pair of studies has examined post-mortem differences in epigenetic regulation of BDNF in suicidal behavior (Keller et al., 2010, 2011). Examination of BDNF in Wernike's area revealed increased BDNF promoter IX methylation with a corresponding decrease in BDNF mRNA and protein levels in suicide subjects (Keller et al., 2010). Despite this finding, no relationship with TrkB promoter methylation and suicide was revealed (Keller et al., 2011). The failure to find epigenetic regulation of TrkB receptor in suicidal behavior may be supported by the lack of consistent findings regarding regulation of TrkB expression in stress-mediated rodent models of PTSD (Smith et al., 1995a,b; Takei et al., 2011). Examination of post-mortem epigenetic modifications in depressed patients who underwent antidepressant treatment has also been associated with a decrease in H3K27 trimethylation at BDNF promoter IV in the PFC and subsequent increased BDNF mRNA. However, no difference in H3K27 was observed within BDNF promoter IV for those patients not on

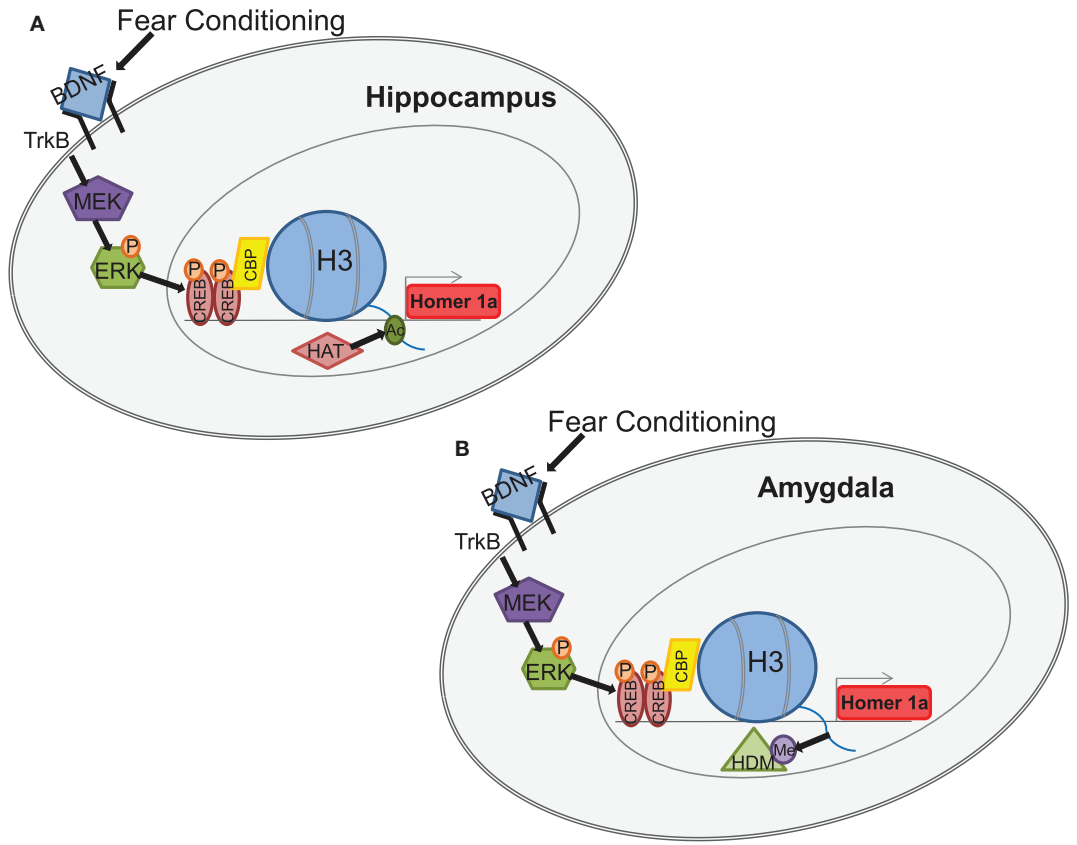

FIGURE 3 | Example of BDNF-mediated transcriptional activation, through epigenetic regulation of the homer1a synaptic plasticity gene in hippocampus and amygdala (Mahan et al., 2012). Fear conditioning rapidly increases BDNF-signaling in both the hippocampus and amygdala and results in activation of the TRKB pathway that activates MEK and ERK. ERK further phosphorylates CREB which is then translocated to the nucleus where it binds to CRE sites in the promoter region of *homer1a*. CREB subsequently recruits CBP which induces specific histone changes **(A)** increased acetylation in the hippocampus and **(B)** decreased methylation in the amygdala, both of which result in increased *homer1a* transcription (Figure courtesy of Amy Mahan, PhD).

antidepressant treatment (Chen et al., 2011). While these studies suggest a correlation between post-mortem epigenetic modifications at BDNF promoters, it is unclear how these differences relate to functional differences in BDNF transcription which underlie depression or suicide behavior *in vivo*.

Despite the suggestion of a role for epigenetic modulation of BDNF activity in depression and suicide, only one study has demonstrated a modest association between increased methylation of one BDNF CpG site with current PTSD status (Smith et al., 2011). Moreover, although previous reports have noted lower levels of BDNF from blood serum in PTSD populations (Dell'Osso et al., 2009; Berger et al., 2010; but see Hauck et al., 2009), it is unclear how this reduction relates to either a risk factor for the development of PTSD or is rather a consequence of PTSD. Despite this preliminary evidence, a recent model relating genetic risk factors associated with impaired fear extinction such as the BDNF val66met polymorphism, potential epigenetic-modifications associated with early-childhood adversity and trauma, and adulthood trauma has emerged to implicate a role for BDNF function in the comorbidity of PTSD and bipolar disorder (Rakofsky et al., 2012). This model, with support from animal studies suggests that there may be a "critical period" in development during which early-life trauma or stress can most readily result in epigenetic modifications on genes associated with stress and PTSD which later impact the likelihood for the future development of PTSD. In support of this model, data have demonstrated epigenetic-modifications associated with genes which regulate GR function in early-childhood adversity and subsequent risk for the development of PTSD. Further, as work has suggested that glucocorticoid exposure can influence BDNF expression (Gourley et al., 2009), it is likely that with further examination of these genes and the interactions amongst them, a role for epigenetic modifications of BDNF may be revealed for PTSD.

EPIGENETIC MODIFICATIONS OF FKBP5 AND GLUCOCORTICOID RECEPTOR FUNCTION

A role for the hypothalamic-pituitary adrenal (HPA) axis in stress and fear memory has been well appreciated (Roozendaal et al., 2009), and one important target of HPA activity is the regulation of GR activity. GR translocation is mediated by the GR co-chaperone protein FK506 binding protein 5 (*FKBP5*) which is associated with the chaperone heat shock protein 90 (hsp90) to form a chaperone complex which regulates GR dynamics (Hubler and Scammell, 2004). Typically *FKBP5* is regulated via a negative feedback loop such that GR activation promotes an increase in *FKBP5*, which limits GR translocation to the nucleus and GR-dependent transcriptional activation (Binder, 2009; **Figure 4**). Many *FKBP5* polymorphisms have been identified and evidence has emerged to suggest that these

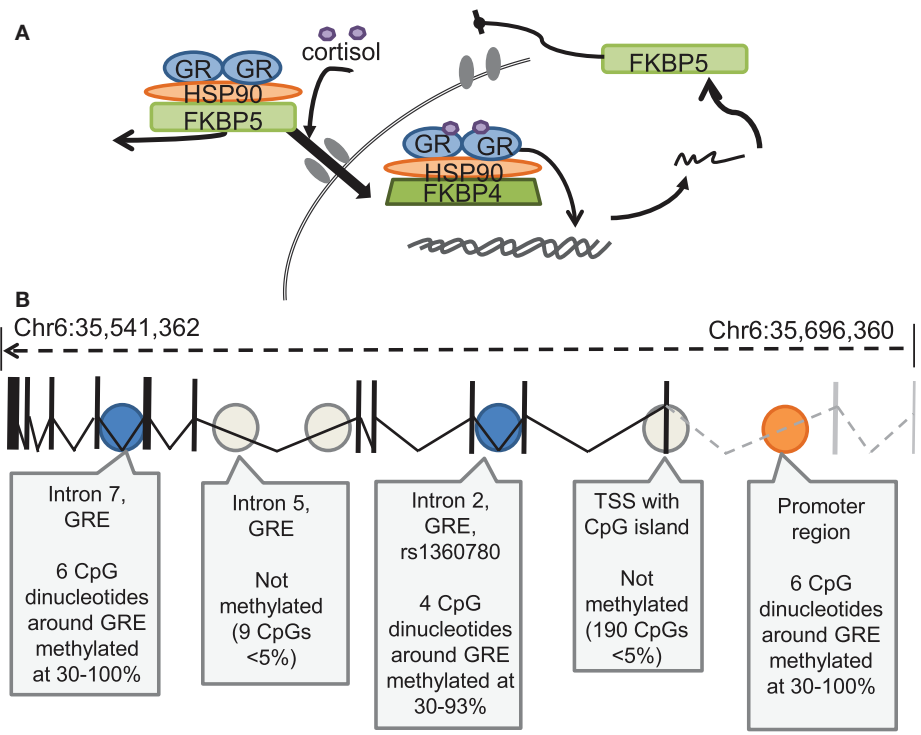

FIGURE 4 | FKBP5 regulation of GR function, and DNA methylation regulatory sites within the *FKBP5* Gene (A) Schematic diagram of FKBP5 regulation of Glucocorticoid Receptor (GR) function. FKBP5 protein acts as an inhibitory chaperone, preventing GR translocation to the nucleus. With increasing cortisol binding, FKBP5 is displaced by FKBP4, allowing for translocation and gene activation, with more FKBP5 mRNA produced as one of the GR-sensitive genes, completing the intracellular negative feedback loop. *(Figure courtesy of Elisabeth Binder).* **(B)** DNA methylation of the FKBP locus, in which significant DNA methylation was observed in the promoter region, Intron 2 and intron 7 of the FKBP5 gene (Figure adapted from Klengel et al., 2013).

polymorphisms impact cortisol response. Such findings suggest that individuals carrying these polymorphisms may have maladaptive responses to stress and thus may be at enhanced risk for developing psychiatric disorders (Ising et al., 2008; Mahon et al., 2010; Touma et al., 2011). Indeed HPA dysregulation and GR resistance, mediated by alterations in the *FKBP5*-governing negative feedback loop, have been discussed as a key endocrine marker for mood disorders (Binder, 2009). In agreement with this hypothesis, a recent study revealed differences in gene expression profile responses in depressed patients versus healthy controls using a dexamethasone-suppression test (Menke et al., 2013), a tool commonly used to measure GR resistance. This study revealed that depressed patients had much higher cortisol levels 21 h following dexamethasone (dex) intake compared to controls, suggesting GR resistance in depressed patients. This is in contrast to the enhanced GR-sensitivity typically seen in PTSD patients. Further, this study explored gene expression profiles from peripheral blood accompanying dex treatment. It was found that depressed patients had reduced levels of *FKBP5* following dex treatment compared to controls, a pattern of findings which suggest impaired GR sensitivity and *FKBP5* expression in depressed patients. Additionally these findings confirm the usefulness of dexamethasone-stimulated gene expression profiles as a biomarker tool to uncover depression-related alterations in GR reactivity.

Animal models of stress have also noted a role for *FKBP5* in coping behaviors and in the mediation of stress effects. Studies employing *FKBP5* knockout mice have demonstrated reduced HPA reactivity and highlighted a role for *FKBP5* in mediating coping behaviors to stress (Touma et al., 2011; Hartmann et al., 2012). Further, a study employing a chronic corticosterone exposure paradigm, previously shown to result in a phenotype similar to chronic stress exposure (Gourley et al., 2009) demonstrated an increase in FKBP5 mRNA in the hippocampus, hypothalamus, and blood of mice with a correspondent decrease in FKBP5 intron 5 DNA methylation (Lee et al., 2010). This group further determined that this chronic corticosterone exposure paradigm also resulted in alterations in FKBP5 methylation, as evident from blood samples which correlated with glucocorticoid load and with anxiety-like behaviors as measured by time spent in the closed arms of an elevated plus maze (Lee et al., 2011). Thus these findings highlight the consequences of prolonged corticosterone exposure on levels of FKBP5 and the ability to correlate epigenetic modifications of *FKBP5* in blood samples with behavioral consequences associated with a stress phenotype. Moreover, FKBP5 mRNA has been shown to be increased following stress, especially in areas already associated with stress and emotional reactivity, including the paraventricular nucleus, central nucleus of the amygdala, and hippocampus, regions which express low basal levels of FKBP5 mRNA (Scharf et al., 2011). However, it is important to note that studies in humans have revealed lower levels of *FKBP5* and GR mRNA in the amygdala of suicide victims (Perez-Ortiz et al., 2012) and lower levels of FKBP5 mRNA in the blood of PTSD patients (Yehuda et al., 2009). These data suggest that further work is needed to address the mechanisms through which shortened stress exposure as is likely in animals models leads to an increase in *FKBP5* expression, while chronic and longer lasting stress, as is likely the case in human suicide victims result in a reduction of *FKBP5*. Despite this potential contradiction, these findings further suggest that *FBKP5* is critical for stress reactivity, and that prior-stress experience may impair levels of *FKBP5* to result in poor adaptation to future stress. Additionally, as was mentioned above, human patients with depression have been consistently found to have GR non-responsiveness and those with PTSD to have GR hypersensitivity. Some of the differences in these studies may actually be due to increased *FKBP5* function associated with GR non-responsiveness, and decreased *FKBP5* associated with GR hypersensitivity. Thus, the apparent variance across studies may be due to our yet incomplete understanding of the differential biology of depression vs. PTSD.

In light of these findings, nice parallels can be seen in work examining *FKBP5* polymorphisms and the epigenetic regulation of *FKBP5* in human populations. First, an association between the *FKBP5* polymorphism rs9470080 and its interaction with early-childhood stress has been found in the risk for development of PTSD in an African American population (Binder et al., 2008; Xie et al., 2010). In support of this finding, distinct *FKBP5* genotypes have been shown to define distinct PTSD subtypes, such that while baseline cortisol levels were found to be reduced in non-risk allele carrying PTSD patients, only patients carrying the *FKBP5* polymorphism risk-allele, rs9296158, show GR hypersensitivity as measured by increased dex suppression (Mehta et al., 2011). Further evidence for a role of *FKBP5* polymorphisms in risk for PTSD development can be observed in a recent study which demonstrated that *FKBP5* genotype and methylation patterns are associated with GR sensitivity and early-childhood trauma exposure (Klengel et al., 2013). The authors found that epigenetic modifications which likely result from early-life trauma enhance *FKBP5* genotype-dependent effects on GR sensitivity and ultimately regulate risk or predisposition for the development of PTSD or depression.

In further support of a role for glucocorticoid-system mediated alterations with PTSD, additional work has revealed a role for epigenetic regulation of the gene encoding the GR *Nr3c1*, and its polymorphism *Bcl1* in fear, stress, and anxiety. A study revealed that suicide victims with a history of early-childhood abuse have differential DNA-methylation patterns around transcription start sites within the *Nr3c1* locus in the hippocampus, whereas no difference was observed between suicide-completers in the absence of early-childhood abuse or control subjects (Suderman et al., 2012). Moreover, suicide victims with a history of child abuse have been found to have reduced hippocampal GR expression and an increase in *Nr3c1* promoter methylation (McGowan et al., 2009). Additionally it has been demonstrated that increased *Nr3c1* promoter methylation levels obtained from peripheral blood samples of bipolar disorder, manic depressive disorder, and PTSD subjects correlate with the severity of childhood abuse (Perroud et al., 2011). These findings are supported by additional studies of DNA methylation of *Nr3c1* where leukocyte DNA-methylation pattern differences associated with early-childhood stress correlate with attenuated cortisol responsiveness following a Dex/CRH test (Tyrka et al., 2012).

Examination of the impact and lasting influence of early-life stress has revealed differential methylation patterns of the *Nr3c1* promoter obtained from peripheral blood samples in mothers and newborns from the Democratic Republic of the Congo (Mulligan et al., 2012). In this region plagued with war, stress-related to ongoing war was most highly correlated with low birth weight. Further it was revealed from umbilical cord blood samples that newborns from stressed mothers have increased levels of *Nr3c1* promoter methylation (Mulligan et al., 2012). These data are the first of which we are aware to show the earliest evidence of epigenetic modifications of the *Nr3c1* gene associated with early-life stress. These findings underlie the utility of these biomarker approaches to identify epigenetic-modifications associated with early-life trauma which may influence susceptibility for the future development of psychopathology including PTSD. Additional support for a role of *Nr3c1* in risk for PTSD comes from a pair of studies demonstrating that individuals with the *Nr3c1* polymorphism *Bcl1* have been shown to have enhanced emotional memory recall (Ackermann et al., 2012), have lower levels of basal plasma cortisol levels, and recall more traumatic memories from time spent in intensive-care units following cardiac surgery (Hauer et al., 2011). However, it is worth noting that a study examining GR polymorphisms in a cohort of Vietnam veterans found no association of genotype with alterations in GR responsiveness, nor was any difference in baseline cortisol level observed between PTSD and non-PTSD veterans (Bachmann et al., 2005).

While no epigenetic modifications to the *Nr3c1* gene itself have been specifically linked to PTSD diagnosis, the mounting evidence for a role of early-life stress in mediating susceptibility for the later development of PTSD, evidence for epigenetic alterations associated with suicide, and data showing early-life stress mediation of epigenetic modifications in *Nr3c1* suggest that a critical link may eventually be revealed. Further evidence for this potential link comes from another study employing a biomarkers approach which demonstrated lower levels of methylation of the *TPR* gene, which has been linked to *Nr3c1* transcription, in individuals with PTSD (Smith et al., 2011).

The human clinical data discussed so far have parallels with animal studies modeling the effects of stress on epigenetic modulation of genes as well as the impact of stress on future experience with trauma. However, there have been some very interesting findings revealing epigenetic modifications solely from human cohorts which are worth mentioning. A series of studies from the Detroit Neighborhood Health Study (DNHS) has revealed a role for epigenetic modifications of *SLC6A3* and *SLC6A4*, genes which encode for dopamine and serotonin transporters, respectively (Koenen et al., 2011; Chang et al., 2012). Individuals with a polymorphism of the *SLC6A3* gene were found to be at risk for the development of PTSD. However, individuals with methylation of this polymorphism were found to be at even greater risk for PTSD (Chang et al., 2012), suggesting that epigenetic alterations in the gene associated with the dopaminergic system may underlie risk for PTSD. Further work from this group established a potential protective role for epigenetic modifications to the *SLC6A4* gene (Koenen et al., 2011). This study determined that *SLC6A4* hypermethylation was associated with protection from the influence of multiple traumas on risk for development of PTSD, suggesting for the first time that epigenetic alterations may be protective against PTSD development. While these data suggest epigenetic-modifications associated with dopaminergic and serotonergic function in both risk and protection from PTSD development, it is important to consider the possibility that epigenetic modifications from a single cohort, such as the DNHS, may have a different epigenetic landscape than other cohorts due to the influence of social and environmental factors which can leave their mark on the epigenome. Therefore, as progress in revealing epigenetic modifications in human cohorts continues, there is a great need to examine the reliability of these modifications across multiple cohorts in order to make the most confident predictions of potential risk factors associated with PTSD development. In agreement with this notion, work from the DNHS has exposed an association between epigenetic alterations of inflammatory and immune response genes with PTSD risk (Uddin et al., 2011a,b), a pattern of findings which has been replicated in an inner-city Atlanta population (Smith et al., 2011) and suggests that an increase in methylation of anti-inflammatory genes, but decrease in methylation for pro-inflammatory genes is associated with PTSD. Thus these data suggest, across cohorts, that inflammation response genes and epigenetic-modifications associated with these genes may underlie PTSD.

FUTURE DIRECTIONS

Great progress has been made in revealing both dynamic regulation of epigenetic modifications including changes in histone acetylation and DNA methylation, which underlie the initial formation of aversive and enduring fear memories in Pavlovian fear paradigms, as well as the enduring epigenetic-modifications associated with early-life stress. While human studies have parallels with these animal studies of early-life stress, very little (if anything at all) is known about the presence of such dynamic epigenetic regulation in humans accompanying the initial formation of traumatic memories. Additionally, while animal and post-mortem studies have enabled the ability to demonstrate epigenetic modifications in the brain accompanying fear memory formation, stress, or trauma exposure, it is unclear how these findings correlate to the recent emergence in biomarkers-based epigenetic approaches.

As highlighted throughout the work presented, some foundational progress has been made in identifying a vital role for the epigenetic mechanisms required for initial fear memory consolidation. Initial findings have also noted a role for epigenetic mechanisms in fear memory reconsolidation and while the translation of reconsolidation-based memory interventions to the clinic is still in its infancy, the emergence of a role for epigenetics in memory reconsolidation opens up an additional set of epigenetic-modifying compounds for use in the clinic in combination with reconsolidation-based therapy. As rodent models of fear extinction learning have found great translational support, these preliminary findings of epigenetic mediation of extinction also propose that epigenetic-modifying compounds may be beneficial for the treatment of fear and anxiety disorders when administered in conjunction with exposure-based psychotherapy. Finally, the translation of

animal models examining epigenetic modifications related to early-life stress into studies examining the epigenetic modifications of genes associated with risk for PTSD supports continued research in the area such that therapeutic approaches targeting these epigenetic modifications may be developed not only as potential prevention options but to expand the current treatments available.

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Interindividual variability in stress susceptibility: a role for epigenetic mechanisms in PTSD

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Post-traumatic stress disorder (PTSD) is a psychiatric condition characterized by intrusive and persistent memories of a psychologically traumatic event that leads to significant functional and social impairment in affected individuals. The molecular bases underlying persistent outcomes of a transient traumatic event have remained elusive for many years, but recent studies in rodents have implicated epigenetic modifications of chromatin structure and DNA methylation as fundamental mechanisms for the induction and stabilization of fear memory. In addition to mediating adaptations to traumatic events that ultimately cause PTSD, epigenetic mechanisms are also involved in establishing individual differences in PTSD risk and resilience by mediating long-lasting effects of genes and early environment on adult function and behavior. In this review, we discuss the current evidence for epigenetic regulation of PTSD in human studies and in animal models and comment on ways in which these models can be expanded. In addition, we identify key outstanding questions in the study of epigenetic mechanisms of PTSD in the context of rapidly evolving technologies that are constantly updating and adjusting our understanding of epigenetic modifications and their functional roles. Finally, we discuss the potential application of epigenetic approaches in identifying markers of risk and resilience that can be utilized to promote early intervention and develop therapeutic strategies to combat PTSD after symptom onset.

Keywords: PTSD, epigenetics, stress, DNA methylation, chromatin, individual differences

Post-traumatic stress disorder (PTSD) is a devastating psychiatric condition that develops in the aftermath of a traumatic life event. It is characterized by persistent and intrusive memories that interfere with daily functioning, often to the point of physical and emotional disability (American Psychiatric Association, 2000). In recent years, epigenetic mechanisms in the central nervous system emerged as the long sought-after link between transient environmental stimuli, including trauma, and persistent changes in gene expression and behavior (Zovkic et al., 2013). Epigenetic mechanisms were initially implicated in behavior through their ability to shape stress- and mood-associated neural and behavioral outcomes caused by early life experiences (Champagne and Meaney, 2001; Meaney, 2001; Fish et al., 2004; Weaver et al., 2004a,b). Shortly after, the epigenetic mechanisms once thought to be stable across the lifespan were found to be dynamically regulated during learning and memory in adult rodents (Levenson and Sweatt, 2005, 2006; Chwang et al., 2006; Levenson et al., 2006; Lubin and Sweatt, 2007; Miller and Sweatt, 2007). This temporal duality implicated epigenetic mechanisms in shaping individual variation in risk and resilience in response to early life experiences and in mediating environmental triggers of psychiatric disorders in adulthood (McGowan et al., 2009; Labonte et al., 2012; Russo et al., 2012; Zovkic and Sweatt, 2013).

Vast individual differences in PTSD prevalence among people exposed to traumatic events (Kessler et al., 1995), the contribution of early life events to adult risk for psychopathology (Yehuda and Bierer, 2009; Kremen et al., 2012; Russo et al., 2012), and the ability of stressors to regulate gene expression (Cullinan et al., 1995) pointed to epigenetic involvement in PTSD before any direct evidence for a link was available (e.g., Yehuda and Bierer, 2009). Soon, human studies provided evidence linking epigenetics with PTSD, either as a consequence of trauma or as a mediator of gene-environment interactions in PTSD risk and resilience (e.g., Ressler et al., 2011; Skelton et al., 2012; Klengel et al., 2013; Norrholm et al., 2013). While comparatively few animal studies have directly investigated epigenetic involvement in PTSD, the related literature on the epigenetics of stress and fear conditioning provides important insights into the role of these mechanisms in establishing persistent fear in response to stressful stimuli (Zovkic and Sweatt, 2013). Thus, one of our goals is to review the relatively small literature that directly addresses the involvement of epigenetics in PTSD and put it into the broader context of epigenetics in stress and fear learning. Given that approximately one in eight individuals who experience traumatic life events develop PTSD (Kessler et al., 1995), we will place particular emphasis on individual differences in PTSD and the need for animal models to consider this variation in studies of risk and resilience. Finally, to encourage direct investigation of epigenetic mechanisms in animal models of PTSD, we will provide a brief technical overview of the rapidly evolving approaches for measuring DNA methylation that can serve as a launching pad for researchers interested in applying these tools to their own work. Overall, we will explore the idea that trauma induces long-lasting

epigenetic changes that mediate PTSD-associated outcomes, and that the exact changes elicited are influenced by individual-specific epigenetic landscape on which the traumatic event occurs.

OVERVIEW OF EPIGENETICS IN PTSD

Post-traumatic stress disorder is a multifaceted disorder that involves the deregulation of stress-responsive endocrine systems and of neurotransmitters and neuromodulators involved in arousal, depression, and anxiety (Baker et al., 2012). Although studies focused on identifying the relative contributions of these various systems have shaped the current understanding of the biological basis of PTSD, further progress will be driven by identifying common factors that drive and maintain their deregulation. Epigenetic modifications are emerging as one such factor because of their role in mediating gene expression for various components of the endocrine system, signaling molecules, and transcription factors (Champagne et al., 2006; Weaver et al., 2007; Chang et al., 2012; Russo et al., 2012; Klengel et al., 2013). In turn, epigenetic modifications are themselves regulated by upstream signaling cascades that converge to mediate epigenetic modifications in the nucleus, whereas existing epigenetic states alter the efficacy of the upstream signaling cascades on gene expression and behavior (Levenson et al., 2004; Fischer et al., 2007; Roozendaal et al., 2010; Graff and Tsai, 2011; Graff et al., 2011). In addition to providing an integrated perspective on psychiatric disorders, this bidirectionality offers promise for epigenetically based pharmacotherapies, which can reduce the threshold for, and amplify the response to, upstream signals (Graff and Tsai, 2011).

EPIGENETIC MECHANISMS

DNA methylation initially gained notoriety for its role in the maintenance of cellular identity and heritable changes in gene expression throughout the cell cycle. DNA is initially methylated at the 5′ position of the cytosine–pyrimidine (5mC) ring by *de novo* DNA methyltransferase enzymes DNMT3a and 3b (Chiang et al., 1996; Turker, 1999; Bird, 2002; Cheng et al., 2010). This pattern of methylation is maintained throughout cell division by the maintenance DNMT1, which methylates cytosines opposite a methylated strand to allow for self-perpetuation of DNA methylation across cell divisions (Santos et al., 2005; Cheng et al., 2010). Given the energetic cost of removing a covalent 5mC bond and its role in the maintenance and propagation of cellular identity, 5mC was classically considered to be an irreversible mark. This notion has held largely true for the maintenance of cell-specific gene expression, but the classical view was challenged by evidence of active DNA demethylation in response to environmental stimuli in the brain, estrogen stimulation in cultured cells, and in muscle after exercise (Levenson et al., 2006; Miller and Sweatt, 2007; Kangaspeska et al., 2008; Metivier et al., 2008; Barres et al., 2012).

Indeed, 1.4% of measured CpGs (cytosines adjacent to guanines) in the granule cells in the dentate gyrus of the hippocampus exhibited altered DNA methylation in response to neuronal activation (Guo et al., 2011a), indicating that active DNA methylation and demethylation are a normal outcome of neuronal activity. Mechanisms of active DNA demethylation have been elusive, with limited success of early efforts to identify enzymes that remove 5mC directly. However, recent studies have provided a flurry of evidence suggesting that active demethylation is a stepwise process in which modifications of 5mC create a base mismatch that is replaced by an unmethylated cytosine through base excision repair (BER) (Wu and Zhang, 2010). Oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) has emerged as a key step in active DNA demethylation in the brain and is accomplished by the ten-eleven translocation (TET) family of methyl-cytosine-dioxygenases (Tahiliani et al., 2009; Guo et al., 2011b; Ito et al., 2011). The resulting 5hmC is further modified by AID/APOBEC deaminases to yield 5 hydroxymethyluridine (5hmU) that is recognized by TDG and MBD4 to generate apurinic/apyrimidinic (AP) sites, which are cleaved by AP endonucleases and subjected to BER for replacement with an unmodified cytosine (5mC) (Ma et al., 2009a,b; Fritz and Papavasiliou, 2010; Bhutani et al., 2011; Guo et al., 2011b; Niehrs and Schafer, 2012; Grayson and Guidotti, 2013). Gadd45 proteins have also been implicated in this process, presumably by linking the AID/APOBEC deaminases with glycosylases to promote BER, which is particularly relevant for activity induced demethylation given the high sensitivity of Gadd45 to neural activity (Ma et al., 2009a,b). The much higher abundance (10-fold) of 5hmC in the rodent and human brain relative to other tissues (Kriaucionis and Heintz, 2009; Munzel et al., 2010; Jin et al., 2011; Szulwach et al., 2011), its increased levels over development (Munzel et al., 2010; Song et al., 2011b), and its regulation by neuronal activity (Guo et al., 2011b), suggest that this modification may be particularly relevant for cognitive function.

DNA methylation was initially identified as a mechanism for persistent repression of gene activity by the recruitment of co-repressor complexes (Karymov et al., 2001; Drewell et al., 2002) and the direct interference of 5mC with binding of transcriptional machinery (Iguchi-Ariga and Schaffner, 1989) to gene promoters. However, recent studies have shown that this is not always the case and that 5mC may at times be associated with gene *activation*. For example, although DNMTs are traditionally implicated in gene silencing (Chen et al., 2002), DNMT3a2 was recently associated with gene activation and improved cognitive function (Kotini et al., 2011) and *de novo* DNMTs may even be involved in DNA demethylation (Chen et al., 2012a). New evidence also suggests that the effect of DNA methylation on transcription may depend on the specific site on which the modification occurs, with 5mC at gene promoters and regulatory elements promoting gene repression and 5mC in gene bodies promoting gene activity (Hellman and Chess, 2007; Zilberman and Henikoff, 2007; Maunakea et al., 2010; Shenker and Flanagan, 2012). Although gene repression has been associated with the methylation of CpG islands in gene promoters, recent studies suggest that only 6.8% of CpGs are found in CpG islands (Rollins et al., 2006) and CpG islands are not ubiquitous to all human promoters (Deaton and Bird, 2011). In fact, newly identified CpG-island flanking regions termed CpG shores exhibit comparatively low CpG density and higher variability in methylation in cancer compared to CpG islands (Irizarry et al., 2009), implicating these sites in gene regulation. It is not clear to what extent these site-dependent functional differences are attributable to 5mC compared to 5hmC, since recent studies have demonstrated that 5hmC is depleted from promoters and intergenic regions and is enriched in gene bodies of actively transcribed cerebellar genes (Mellen et al., 2012). In contrast, a 5hmC peak that

appeared 900 bp 5′ of the transcription start site was not correlated with transcription (Mellen et al., 2012), perhaps suggesting an alternative function. Of note, 5mC in the latter study was depleted from the bodies of actively transcribed genes and there was a tendency for a negative association between intragenic 5mC and gene expression in some cell types more than others (Mellen et al., 2012). Overall, however, intragenic 5hmC:5mC ratio was a better predictor of gene expression than 5mC or 5hmC alone (Mellen et al., 2012), pointing to the importance of considering both modifications in relation to transcription in different cell types. More broadly, these data indicate that the role for DNA methylation is more heterogeneous than initially suspected and that further studies will be required to fully understand the relationship between DNA methylation and transcription.

Many effects of DNA methylation are exerted through interactions with DNA-binding factors that recruit co-activators or co-repressors to mediate gene transcription and post-translational modifications (PTMs) of histones (Strahl and Allis, 2000; Ooi et al., 2007; Borrelli et al., 2008). DNA is packaged into nucleosomes, which are the building blocks of chromatin and are composed of ∼147 bp of DNA wrapped around an octamer of 2 each of histones H2A, H2B, H3, and H4 (Quina et al., 2006). Histones are critical regulators of DNA accessibility and chromatin compression or openness is determined in part by PTMs of histones (Strahl and Allis, 2000). Histones contain protruding tails that can be modified in a number of ways, most notably through acetylation, methylation, and phosphorylation (Strahl and Allis, 2000). As with DNA methylation, the pattern of histone modifications appears to differ based on the genomic region of interest, notably between introns and exons and around the transription start site (TSS) (Lieb and Clarke, 2005; Zilberman and Henikoff, 2007; Choi et al., 2010; Huff et al., 2010).

Of these various chromatin modifications, histone acetylation has received the most attention and is typically involved in the activation of gene expression (Mujtaba et al., 2007), whereas histone methylation can be involved in either activation or repression (Nakayama et al., 2001; Peters and Schubeler, 2005). Histone acetylation is catalyzed by histone acetyltransferases (HATs) and the reverse deacetylation reaction is performed by a large number of histone deacetylases (HDACs) (Hebbes et al., 1988; Wade, 2001). The binding of these enzymes is influenced by DNA methylation and associated methyl-CpG binding proteins, particularly MeCP2, which recruits HDACs to gene promoters to facilitate histone deacetylation (Lorincz et al., 2004; Liu et al., 2005). Recent evidence from the model plant *Arabidopsis thaliana* and from treatment with the HDAC inhibitor (HDACi) valproic acid (VPA) suggests that the reverse reaction may also be true, with HATs participating in DNA demethylation (Tremolizzo et al., 2002, 2005a,b; Detich et al., 2003; Qian et al., 2012). Indeed, the methyl-CpG binding protein MeCP2 interacts with CREB to promote histone acetylation (Chahrour et al., 2008), although it is not clear whether this relationship occurs through MeCP2 interactions with 5hmC and 5mC (Mellen et al., 2012).

MeCP2 binds to both 5mC and 5hmC in the cerebellum, but binding at 5hmC was associated with accessible chromatin and gene activation, whereas binding at 5mC was associated with closed chromatin and gene repression (Mellen et al., 2012). These effects were mediated by different residues on MeCP2, with a mutation in R133C preferentially altering binding to 5hmC compared to 5mC (Mellen et al., 2012). Other have also reported chromatin-regulating effects of neurally expressed MeCP2, with a weak association of MeCP2 with open chromatin and a strong association with closed chromatin (Thambirajah et al., 2012). Overall, there is some overlap in DNA-binding factors that interact with 5mC and 5hmC, but each modification also has unique interacting partners and different affinities for certain MBDs and other DNA-binding proteins (Hashimoto et al., 2012; Spruijt et al., 2013). These data suggest that 5hmC may be a transcriptional regulator in its own right rather than having a sole function as an in intermediary in active DNA demethylation.

The rapid advancements in techniques for detecting 5mC and 5hmC (see **Box 1**) and the application of whole-genome sequencing approaches have led to an unprecedented rate of growth in our understanding of epigenetic mechanisms in the regulation of gene expression. The wealth of new information has challenged many of the classical views of epigenetics in general and DNA methylation in particular, with many important implications for interpreting past and future data in the field. The most direct lesson from recent studies is that epigenetic modifications have diverse roles in gene regulation and that their effect on gene expression cannot be directly inferred from examining epigenetic modifications alone.

Although we have focused heavily on DNA methylation, even the typically activating histone acetylation mark has been found on bivalent promoters that can be either activated or repressed (Lin et al., 2007; Vastenhouw and Schier, 2012) and HATs and HDACs tend to colocalize on the same genomic sites (Peserico and Simone, 2011). Similarly, in addition to their role in oxidation of 5mC to 5hmC, TET proteins may have additional regulatory roles, as exemplified by TET1 interaction with a repressive complex that might have a role in gene repression independent of its role in DNA demethylation (Bhutani et al., 2011; Ito et al., 2011; Wu and Zhang, 2011; Williams et al., 2012).

Another critical point is that many of the earlier studies did not distinguish between 5mC and 5hmC, making it difficult to draw inferences regarding the relative contribution of each. Although many approaches have been developed to differentiate the two, many techniques still encompass both modifications (see **Box 1**). Finally, growing evidence for distinct functions of DNA methylation at different genomic sites indicates that the common approach of using a single primer per gene may not be sufficient to detect changes in DNA methylation, or that the direction of the change may depend on the region studied (i.e., intragenic vs. promoter; CpG island vs. CpG-island shore). One solution to this approach is to do whole-genome sequencing, although a cost effective alternative is to utilize multiple primers to represent different regions relevant for detecting functional effects. An additional confound in neurobiology is the difficulty in distinguishing between epigenetic modifications occurring in neurons and glia, which will require more wide-spread use of cell sorting approaches and neuron-specific labels to improve our understanding of epigenetic changes in these distinct cell types.

Box 1 | Review of tools for measurement of hydroxymethylation.

Global 5hmC analysis

Several options exist for the global quantification of nucleotide variants implicated in epigenetic regulation, which include 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), respectively. Currently, various antibodies raised against these epigenetic markers are available (Inoue et al., 2011; Ito et al., 2011) and have been employed in combination with either an enzyme-linked immunosorbent assay (ELISA) or dot-blot analysis for global detection (Koh et al., 2011; Li and Liu, 2011). In addition, some have taken advantage of the enzyme T4 phage β-glucosyltransferase (T4-GβT) and its ability to specifically add glucose to 5hmC to generate β-glucosyl-5-hmC (5gmC) (Terragni et al., 2012). Using this process, a radio-labeled glucose can then be transferred to 5hmC for sensitive and accurate detection of its global levels (Lian et al., 2012).

Another strategy to identify nucleotide modifications involves the use of thin layer chromatography (TLC) coupled with radio labeling. This approach provides a high level of sensitivity and was implemented by several labs leading to the initial discoveries of both 5hmC (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009) and 5caC (He et al., 2011; Ito et al., 2011), respectively. However, of all the methods mentioned above, the gold standard remains the use of liquid chromatography (LC) followed by MS to resolve and precisely measure nucleosides containing these epigenetic marks (Globisch et al., 2010; Munzel et al., 2010; Ito et al., 2011).

Loci specific analysis and 5hmC enrichment

hMeDIP: A number of methods are now available for the measurement of 5hmC levels at the resolution of individual genomic loci. The first, hydroxymethyl-DNA immunoprecipitation (hMeDIP) requires the enrichment of 5hmC-containing DNA, typically with an antibody raised against 5hmC or its sodium bisulfite converted derivative, 5-methylsulfonate (CMS) (Pastor et al., 2011). Alternatively, enrichment can be achieved through T4-GβT-mediated conversion of 5hmC to 5gmC, followed by incubation with J-binding protein-1 (JBP-1); a protein shown to have a strong affinity for the modified base (Robertson et al., 2011). Likewise, some groups have gone on to process 5gmC even further through oxidation and biotinlyation steps (Pastor et al., 2011) (GLIB) or with selective chemical labeling (Song et al., 2011a) (hMe-Seal), allowing for the enrichment of biotinylated 5hmC residues using streptavidin beads. Using either the GLIB or hMe-Seal strategies allow for improved hMeDIP fidelity as well as the enrichment of genomic regions containing low amounts of 5hmC.

Restriction enzyme digestion: Recently, a number of restriction enzymes whose activity is blocked either by 5hmC itself or after 5hmC glycosylation have been characterized (Zheng et al., 2010; Cohen-Karni et al., 2011; Davis and Vaisvila, 2011; Kinney et al., 2011; Song et al., 2011b; Szwagierczak et al., 2011; Wang et al., 2011; Xu et al., 2011). The latter of these enzymes have been termed glucosyl-5-hydroxymethylcytosine sensitive restriction endonucleases (GSREs) and several companies now offer commercial kits to investigators. Due to their unique properties, these enzymes can be used to identify specific 5hmc-containing loci provided the region of interest contains the enzymes recognition site. Difficulties arising due to lack of compatible restriction endonuclease recognition sites in genomic regions of interest may be overcome using several different GRSEs. This technique has been further extended using several GSREs in combination, which enables the determination of both 5hmC and 5mC content at the same genomic location (Davis and Vaisvila, 2011).

Both techniques outlined here are amenable for analysis using quantitative PCR analysis for 5hmC detection at individual loci or for whole-genome profiling, microarray analysis, and next generation sequencing.

Single-base resolution sequencing

Although hMeDIP and GRSE analysis can both be useful tools for the analysis of 5hmC distribution on a genome-wide scale, they are limited in their resolution due to DNA fragmentation size and capture technology. To overcome these limitations, several groups have developed techniques to achieve the sequencing of 5hmC residues at a single-base resolution. The first involves the use of third-generation sequencing technology called single-molecule, real-time (SMRT) sequencing which monitors the incorporation of individual nucleotides by DNA polymerase in real time (Eid et al., 2009) and can detect DNA base modifications like 5mC and 5hmC compared to unmodified cytosine due to changes in DNA polymerase kinetics (Flusberg et al., 2010). This technique has now been combined with hMe-Seal to chemically label 5hmC in an effort to distinguish it further from 5hmC and C during DNA polymerization (Song et al., 2012). A second method termed oxidative bisulfite sequencing (oxBS-Seq) involves comparison of a traditional bisulfite sequenced sample (BS-seq) to that which has undergone oxidative conversion of 5hmC to 5fC, prior to bisulfite conversion (Booth et al., 2012). While BS-seq can convert both 5mC and 5hmC to cytosines (Cs), oxBS-Seq only allows for conversion of 5mC sites to Cs. Thus, the amount of 5hmC present at a particular nucleotide position can then be determined by subtraction of the oxBS-Seq sample from that of the BS-Seq sample. Finally, Tet-assisted bisulfite sequencing (TAB-seq) is a technique which combines the protection of 5hmC by T4-GBT glycosylation with Tet-mediated oxidation of 5mCs to 5caCs (Yu et al., 2012). After bisulfite conversion all 5caCs are converted to Ts, leaving only protected 5hmC residues as Cs, resulting in a straight-forward interpretation of 5hmC data at a base-by-base resolution.

ANIMAL MODELS

Well-designed animal models are critical for uncovering epigenetic mechanisms of PTSD by allowing for the control of the temporal parameters needed to understand predisposition, onset, maintenance, and treatment of the disorder. An appropriate animal model must ensure face and etiological validity by mimicking the conditions of the disorder seen in human patients, which primarily involves exposure to a traumatic event in the case of PTSD (van der Staay et al., 2009). The traumatic event has been modeled in a number of ways, including exposure to an aversive shock in fear conditioning, exposure to a predator, social defeat, restraint, or underwater holding (Adamec and Shallow, 1993; Richter-Levin, 1998; Cohen et al., 2000a,b; Siegmund and Wotjak, 2007; Zoladz et al., 2008; Zovkic and Sweatt, 2013). Given the association between trauma and symptom severity in human patients (Koenen and Uddin, 2010; Uddin et al., 2010), stimuli that can deliver graded levels of trauma are preferred in an animal model (Siegmund and Wotjak, 2007). Shock delivery in fear conditioning is particularly amenable to manipulating gradations in stimulus intensity and duration (Siegmund and Wotjak, 2007),

although different degrees of trauma intensity can also be achieved in predator exposure models by exposing rodents to cat odor alone, a caged cat, or to a direct encounter with a cat (Zoladz et al., 2008; Goswami et al., 2012). The etiological validity of these models can be further improved by combining the traumatic experience with additional risk factors that further enhance vulnerability to PTSD, such as the lack of social support and persistent exposure to chronic mild stress (Zoladz et al., 2008). In one example, the number of rodents expressing PTSD symptoms was increased by combining predator odor exposure with 31 days of social instability stress in an effort to mimic the lack of social support seen in individuals vulnerable to PTSD (Zoladz et al., 2008).

As an extension of the face validity criterion, animal models must include dependent variables that parallel the diverse symptoms of PTSD in order to generate testable predictions for research and treatment in human patients (van der Staay et al., 2009). PTSD patients exhibit a wide range of symptoms, including hyperarousal, increased startle, emotional blunting, and social withdrawal, which can be included as outcome variables in response to a variety of traumatic stimuli (Siegmund and Wotjak, 2007; Uddin et al., 2010; Skelton et al., 2012). These symptoms are induced by fear conditioning and predator exposure models of PTSD and are typically assessed after a prolonged stress-free period (Zovkic and Sweatt, 2013). For example, fear conditioning can be used to assess intensity-dependent freezing upon re-exposure to the training context to test for the expression of associative fear and upon exposure to a novel, neutral tone to provide an index of non-associative fear sensitization, hyperarousal, and startle (Siegmund and Wotjak, 2007). Importantly, the expression of sensitized fear to a novel auditory cue was found to increase with time after shock exposure (Siegmund and Wotjak, 2007), which is particularly important in light of the proposed revisions to DSM-V that emphasize delayed symptom onset over the initial fear response (Resick and Miller, 2009; Friedman et al., 2011a,b). Indeed, many patients who are diagnosed with PTSD show little to no emotional response upon initial exposure to trauma, with symptoms developing only with the passage of time (Shalev et al., 2000; Griffin, 2008; Friedman et al., 2011a,b). Associative fear is particularly amenable to delayed behavioral assessment (Miller et al., 2010) and has the advantage of modeling the re-experiencing of fear in PTSD patients by presenting animals with an aversive cue or context without the need for re-exposure to the traumatic stimulus (i.e., footshock) (Zovkic and Sweatt, 2013).

MODELS OF PREDISPOSITION TO PTSD

A difficulty with PTSD studies in humans is the poor ability to distinguish between pre-existing risk factors and trauma-induced outcomes. Co-twin studies, in which only one twin has experienced trauma, found that certain parameters that were thought to be caused by trauma may actually be pre-existing risk factors for PTSD, including impaired cognitive function and reduced hippocampal volume (Kremen et al., 2012). It can also be difficult to separate out epigenetic outcomes from epigenetic risk factors in non-controlled studies of human patients, although cumulative effects of trauma on DNA methylation of immune system genes suggest that traumatic experiences are key drivers of epigenetic outcomes in this scenario (Uddin et al., 2010).

Animal models can be particularly valuable in this regard by evaluating temporal parameters of variables identified in human studies to determine their relative role in conferring risk for PTSD or outcomes of trauma. An evaluation of individual differences is a key component for the success of such models (Yehuda and Bierer, 2009). The emphasis of animal models of PTSD tends to be on the stress exposure (induction) and the associated depressive and anxious phenotypes (read out), which are valuable tools for defining the cognitive, molecular, and neuroanatomical outcomes of stress exposure, but are less productive in explaining the relatively low incidence of persistent psychopathology in response to trauma (Yehuda et al., 2006). Individual differences in rodents can be investigated by classifying animals according to natural variation in behavior, by utilizing genetic predictors of risk and selective breeding strategies based on traits associated with risk and resilience (Scharf and Schmidt, 2012). Cohen and Zohar (2004) developed a model of classifying rodents according to natural variation in responses to predator exposure, in which ∼22% of the rats meet the PTSD criteria based on behavioral, endocrine, and sympathetic markers. The *ad hoc* classification system used in these studies is extremely useful for understanding long-term adaptations in behavioral and molecular systems that distinguish vulnerable and resilient individuals after trauma, but such models do not provide any information on the source of vulnerability or resilience before trauma (Siegmund and Wotjak, 2007; Zovkic and Sweatt, 2013). Selective breeding of vulnerable and resilient mice can help circumvent this problem by allowing for the assessment of pre-existing differences prior to trauma (Siegmund and Wotjak, 2007). For example, strain differences have been noted between PTSD vulnerable B6N and the resilient B6JOla mice, with B6N exhibiting blunted affect and reduced sociability in the absence of any exposure to trauma (Siegmund and Wotjak, 2007). Genetic mouse models can also be used to evaluate the role of risk genes identified in human studies and these models can be combined with developmental stressors to probe for gene-environment interactions at distinct stages of development.

History of stress exposure, particularly in early life, is another essential factor in predisposing individuals to PTSD in adulthood (Scharf and Schmidt, 2012). Although most rodent studies of early environment do not relate behavioral and biochemical outcomes to PTSD directly, their relevance to PTSD has been widely recognized because of the emphasis on the stress-responsive neural circuitry and the related behavioral outcomes (Yehuda and Bierer, 2009).

A widely studied model utilizes variation in maternal licking and grooming (LG) and arched back nursing (ABN) of pups as an index of early life experience. In this model, the offspring of high LG-ABN mothers are less anxious and responsive to stressors compared to the offspring of low LG-ABN dams (Weaver, 2009). However, the extent to which one maternal style is "better" than the other is not clear, as the offspring of high and low LG mothers may each be better adapted to different environmental circumstances (Bagot et al., 2009). However, the offspring of low LG mothers are phenotypically similar to the pups that underwent maternal separation stress (≥3 h separation) and high LG offspring are similar to those who underwent a milder brief separation (15 min), often referred to as handling, in the first 7–14 days of life (Anisman et al.,

1998; Francis et al., 2002; Wilber and Wellman, 2009), indicating that low LG-ABN is akin to a more stressful upbringing. Utilization of maternal maltreatment models with an unambiguous stress exposure in early life (Roth and Sweatt, 2011) will be particularly useful in clarifying the association between stressful upbringing and behavior. Moreover, combining these early life models with adult models of PTSD (Cohen and Zohar, 2004) can provide critical information regarding the interaction between early life experience and the later risk and resilience for PTSD.

PTSD AND THE HPA AXIS

THE HPA AXIS

Many studies of individual differences in risk and resilience have focused on the hypothalamic-pituitary-adrenal (HPA) axis because of its central role in adaptation to stress. The diagnosis of PTSD requires that symptom onset be preceded by a traumatic event and PTSD was recently reclassified as a traumatic and stress-related disorder instead of an anxiety disorder in the newly released DSM-V (Friedman et al., 2011a,b). Stressful encounters induce the release of corticotropin releasing hormone (CRH) from the hypothalamus into the pituitary gland to stimulate the release of adrenocorticotropin hormone (ACTH) and activate the release of glucocorticoids (GCs; cortisol in people, corticosterone in rodents) from the adrenals (Novak et al., 2013). The acute GC response allows individuals to deal with stressors by mobilizing resources, adjusting heart rate, and regulating immune function in the short term, whereas deregulation of GC release over prolonged time periods is detrimental to health, mood, and cognition (Sorrells and Sapolsky, 2007).

GC receptors (GR) are found throughout the brain and are particularly abundant in the hippocampus, where they influence memory formation, cognitive function, and initiate negative feedback to reinstate baseline GC levels after stress (Novak et al., 2013). The HPA axis is a critical regulator of the response to trauma in adults and the deregulation of this response has been implicated in the development of PTSD (Yehuda et al., 2004, 2009). PTSD patients generally exhibit reduced levels of GCs, increased negative feedback, abnormal GR expression, and higher levels of CRH compared to non-traumatized controls (Yehuda et al., 2002; Rohleder et al., 2004; de Kloet et al., 2008), indicating that a blunted GC response to stress may be a risk factor for PTSD (Yehuda and McFarlane, 1995; Yehuda and LeDoux, 2007).

Exposure to GCs stimulates a range of epigenetic modifications that are though to be mediated by the GR (Roozendaal et al., 2010; Yang et al., 2012). The GR is a transcriptional regulator that translocates to the nucleus upon ligand binding, where it activates or represses transcription through actions on the glucocorticoid response element (GRE) (Cairns et al., 1991). According to some estimates, GCs regulate between 1000 and 2000 genes in cortical neurons (Kino, 2007; Sun et al., 2008) and chronic stressors regulate gene expression and histone modifications in neural regions associated with cognitive function, affect, and motivation (Tsankova et al., 2004, 2006; Covington et al., 2011a,b). Effects of GR on transcription are closely tied to epigenetic regulation, as exposure to stress or treatment with glucocorticoids results in the persistent and gene-specific demethylation of GREs in peripherally derived human cells and the rodent brain (Unternaehrer et al., 2012; Yang et al., 2012; Klengel et al., 2013). In addition, exposure to stress in early life alters the expression of epigenetic regulators themselves, as evidenced by increased levels of DNMT3a in the placenta and the brain of pups of stressed mothers (Jensen Pena et al., 2012). Moreover, maternal maltreatment and natural variation in maternal care induce modifications of DNA methylation and expression of target genes that persist into adulthood in the rodent brain (Weaver et al., 2002; Zhang et al., 2006; Roth and Sweatt, 2011). In addition to the direct effects of GR in the nucleus, the GR mediates histone acetylation at target genes indirectly through the activation of the PKA-pCREB/CBP pathway via its membrane-bound receptor (Roozendaal et al., 2010). Overall, these studies demonstrate the ability of stressors to regulate epigenetic modifications, presumably through the actions of GCs on the GR receptor, although other potential pathways, such as sympathetic and noradrenergic responses, cannot be ruled out (Geracioti et al., 2001; Bierer et al., 2006; Videlock et al., 2008).

In addition to regulating epigenetic modifications, the HPA axis is itself epigenetically regulated, particularly during development, when the GR, CRH, and the GC inactivating enzyme *HSD11B2* exhibit persistent alterations in DNA methylation and gene expression in response to maternal stress and natural variation in maternal behavior (Weaver, 2009; Roth and Sweatt, 2011; Jensen Pena et al., 2012). It has been shown, for example, that maternal deprivation between postnatal days 2 and 13 leads to hypomethylation of the cyclic AMP-response element (CRE) in the CRH promoter in the hypothalamus (Chen et al., 2012b), which is particularly relevant given the higher levels of CRH in patients with PTSD (de Kloet et al., 2008). Similarly, offspring of low LG mothers exhibit higher GR methylation, reduced GR expression, and higher reactivity to stress and anxiety in adulthood compared to the offspring of high LG mothers (Weaver, 2009). This effect is established in the first 7 days of life, when high levels of LG induce increased NGFI-1 expression, an upregulation of histone acetylation, and a decrease in DNA methylation at the NGFI-1 binding site of the GR promoter (Weaver et al., 2002, 2004a; Weaver, 2009). Extensive individual differences in DNA methylation of the GR promoter have been reported in people and include increased methylation of the NGFI-1 binding site on the GR promoter and decreased GR expression in the hippocampus of suicide victims with a history of childhood abuse compared to non-abused suicide victims or controls (McGowan et al., 2009).

It thus appears that elevated activity of DNA-binding molecules, such as GR and NGFI-I, may regulate binding to their respective response elements by reducing DNA methylation at the appropriate sites. This relatively well characterized system of maternal regulation of GR expression illustrates the close bidirectional relationship between epigenetic mechanisms and upstream signaling cascades. Moreover, the observation that persistent epigenetic changes are established in the first 7 days of life (Szyf et al., 2007) suggests that long-lasting effects of stress, maternal behavior, and GC exposure are amplified during sensitive periods of development, although the GR promoter is also subject to epigenetic regulation in adulthood (Witzmann et al., 2012).

Depending on the type of maternal care, epigenetic programing of GC release and GR expression may promote vulnerable or resilient phenotypes in adulthood (Dudley et al., 2011). On

the surface, some parallels can be drawn between high LG mothers and the children of Holocaust survivors who had lower levels of GCs and rated their mothers as overprotective (Yehuda and Bierer, 2009). Based on such evidence, Yehuda and Bierer (2008) argued that reduced GC levels in high LG offspring parallel reduced cortisol, increased lymphocyte GR expression, and increased GR sensitivity seen in human PTSD patients (Yehuda et al., 1998, 2002; Yehuda, 2002a,b), although others interpret the reduced cortisol response to stress as protective (Barha et al., 2007). However, there are also reports of reduced levels of GR in leukocytes of PTSD patients (de Kloet et al., 2007), which parallels the reduced levels of GR density seen in low LG offspring. Furthermore, prenatal maternal depression/anxiety leads to increased methylation within the GR promoter at a predicted NGFI-A binding site in infants (Oberlander et al., 2008), likely leading to an overall reduction in GR expression. It is difficult to draw comparisons based on circulating GC and peripheral GR levels alone and studies of GR density in post-mortem brains of PTSD patients are needed to draw clearer parallels. The contention that the offspring of high LG mothers exhibit parallel HPA axis activity to PTSD patients (Yehuda and Bierer, 2009) is consistent with the view that some stress exposure at distinct stages of development confers resilience to PTSD in adults through "stress inoculation" (Ricon et al., 2012). Given the phenotypic similarities between outcomes associated with maternal separation stress and low LG offspring (Anisman et al., 1998; Francis et al., 2002; Wilber and Wellman, 2009), the concept of protective effects of "stress inoculation" (Ricon et al., 2012), and the adaptive function of an appropriate GC response to stress (Yehuda and McEwen, 2004), some have suggested that low LG offspring are actually better adapted to deal with stressful environments than high LG offspring (Bagot et al., 2009).

GENE × ENVIRONMENT × EPIGENOME INTERACTIONS

Individual differences in adult risk and resilience to PTSD are shaped by a combination of genetic and environmental factors. Although genes and environment can produce shifts in risk and resilience on their own, the strongest phenotypic effects are associated with gene-environment interactions that are mediated by epigenetic modifications (Caspi and Moffitt, 2006; Chertkow-Deutsher et al., 2010; Dudley et al., 2011; Russo et al., 2012; Klengel et al., 2013; Zovkic and Sweatt, 2013). Environmental factors are particularly influential during early life, when they interact with epigenetic mechanisms to lastingly alter the expression of a broad range of genes (Sundermann et al., 2012, 2013).

Studies of stress exposure in rodent strains with differential susceptibility to risk and resilience have reported distinct epigenetic and behavioral outcomes, although the specific genes involved in mediating these effects have not been identified. For example, vulnerable BALB/c (BALB) mice exhibited differences in H3 acetylation and DNA methylation of the glial cell-derived neurotrophic factor (GDNF) in the nucleus accumbens compared to the resilient C57BL/6 (B6) mice (Uchida et al., 2011). In addition, BALB mice showed a specific increase in MeCP2-HDAC2 association and B6 showed an increase in MeCP2-CREB association at the GDNF promoter, which were associated with increased and decreased GDNF expression in B6 and BALB mice, respectively. Vulnerability and resilience were also associated with opposite changes in DNA methylation of the same genes (Elliott et al., 2010), and with different levels of intronic DNA methylation and expression of *dlgap2*, a gene coding for PSD95 (Chertkow-Deutsher et al., 2010). In contrast, rodents classified as vulnerable or resilient based on behavioral responses to acute unavoidable stress exhibited differential regulation of distinct genes, with upregulation of neuroendocrine-related genes, growth hormone, and prolactin genes in vulnerable rats, and the downregulation of interferon-β and leukemia inhibitory factor in the frontal cortex of resilient rats (Benatti et al., 2012). Furthermore, Oh et al. (2013) found that an adverse maternal environment in rodents was associated with differentially methylated sites that clustered in the bodies of genes associated with cell adhesion and neurotransmitter receptors in the hippocampus. Finally, a study investigating the effects of environmental stress in non-human primates found an association between increased stress reactivity and higher overall methylation (Kinnally et al., 2011), which led them to speculate that the overall increase in methylation constrained plasticity through gene repression. However, the degree to which this hypothesis will hold true in light of evidence for an activating role of DNA methylation remains to be determined. Overall, these studies demonstrate that similar environmental experiences can produce extremely different outcomes through epigenetic interactions with individual-specific factors that mediate vulnerability and resilience.

THE HPA AXIS

Studies of human patients have found genotype-specific effects of early environment on adult risk for PTSD to be mediated by epigenetic modifications of genes associated with the HPA axis (Koenen and Uddin, 2010; Ressler et al., 2011; Klengel et al., 2013). *Fkbp5* is a negative regulator of GR activity that has received much attention in this regard, in part because of reduced *Fkbp5* and cortisol levels in the patient population (Yehuda and Bierer, 2009; Yehuda et al., 2009; Koenen and Uddin, 2010; Klengel et al., 2013). Single nucleotide polymorphisms (SNPs) in the *Fkbp5* gene are associated with predisposition to PTSD, wherein the same allele confers either risk or resilience in the presence or absence of childhood adversity, respectively (Xie et al., 2010). Specifically, SNPs around the GRE in the human *Fkbp5* gene increased risk for PTSD only in individuals who experienced childhood trauma (Binder et al., 2008; Yehuda et al., 2009). This effect was epigenetically mediated, in that GC exposure was associated with greater *Fkbp5* GRE demethylation and higher *Fkbp5* expression in carriers of the risk compared with the protective allele (Klengel et al., 2013). These data are in contrast to reports of reduced *Fkbp5* expression in PTSD patients (Yehuda et al., 2009), but the discrepancy may in part be explained by variability across peripherally derived cell types and the sorting of data according to genetic variation.

PAC1R, a receptor for PACAP (pituitary adenylate cyclase-activating peptide), is another gene involved in stress responsiveness that has been implicated in PTSD (Dias and Ressler, 2013). PACAP is elevated in the blood of female, but not male, patients with PTSD and an SNP in the estrogen response element of the PAC1R gene interacts with trauma to mediate risk for PTSD in affected individuals (Ressler et al., 2011). Moreover, DNA methylation of the PAC1R gene is positively associated with

PTSD (Ressler et al., 2011), again emphasizing the role of epigenetic factors as mediators of gene-environment associations. These data provide critical insights into the factors involved in establishing vulnerable phenotypes during development, but comparisons of epigenetic responses in vulnerable and resilient adult rodents are required to understand how traumatic experiences produce different outcomes in those individuals.

SYNAPTIC PLASTICITY AND NEUROTRANSMITTER GENES

In addition to the HPA axis, gene-environment-epigenome interactions have been reported for neuromodulators, neurotransmitters, and other molecules relevant for synaptic function and plasticity. For example, the serotonergic system is of clear relevance to PTSD given its well documented, albeit complex role in the regulation of emotional circuitry and fear learning (Rainnie, 1999; Koponen et al., 2005; Christianson et al., 2010), which has prompted an interest in polymorphisms of the serotonin transporter gene *SLC6A4*. A recent meta-analysis found that a short allele with an SNP in the 5-HTT promoter was associated with reduced transcriptional efficiency and increased sensitivity to stress (Karg et al., 2011). As with *Fkbp5*, association of the risk allele with PTSD occurs primarily through interactions with environmental experiences (Kilpatrick et al., 2007; Koenen et al., 2009; Xie et al., 2009), although some discrepancies have been reported (Mellman et al., 2009; Koenen et al., 2011). Epigenetic mediation of gene-environment interactions in PTSD has also been reported for other genes related to synaptic function, such as genes coding for Neuropeptide Y (NPY), BDNF, and molecules involved in the noradrenergic and dopaminergic systems (Boulle et al., 2012; Russo et al., 2012; Skelton et al., 2012; Wu et al., 2013).

In contrast to *Fkpb5* and *SLC6A4,* carriers of the 9R allele of the dopamine transporter gene *SLC6A3* were at greater risk of developing PTSD compared to 10R/10R allele carriers irrespective of environmental factors (Chang et al., 2012), highlighting the independent contribution of certain genes and the need to consider environmental interactions on a gene-by-gene basis. Furthermore, the risk for PTSD was highest in 9R allele carriers who also had high promoter methylation (Chang et al., 2012), indicating that epigenetic factors may mediate the contribution of genetic risk factors, irrespective of environmental factors.

It is important to note that the studies discussed thus far have primarily utilized a candidate-gene approach that allows for a thorough assessment of polymorphisms in a single gene, but is not suited for identifying novel genetic candidates involved in mediating risk and resilience. A recent genome-wide association study (GWAS) of trauma-exposed individuals identified a single polymorphism in the gene encoding the retinoic acid orphan receptor A (RORA), to be associated with PTSD after applying stringent genome-wide significance criteria (Logue et al., 2012). The high-risk SNP was associated with increased risk for PTSD in response to lower levels of trauma compared to individuals with the protective allele. RORA is a member of the NR1 subfamily of nuclear hormone receptors with neuroprotective functions (Jolly et al., 2011), which led the authors to hypothesize that genetically mediated differences in RORA levels are associated with an impaired stress response (Logue et al., 2012). Studies such as this will be critical for identifying novel candidates for PTSD risk and resilience in distinct populations.

EPIGENETIC MEDIATION OF RISK AND RESILIENCE

Although genetic factors are essential mediators of risk and resilience, epigenetic mechanisms also mediate the effects of severe trauma independently of predisposing factors (Chang et al., 2012). Indeed, the extent of epigenetic modifications observed in patients is amplified by the severity and the number of traumatic events experienced (Uddin et al., 2010; Koenen et al., 2011) and distinct methylation profiles of the serotonin transporter gene are differentially associated with risk and resilience even after controlling for genotype (Koenen et al., 2011). Furthermore, observations of altered DNA methylation in response to childhood abuse and bullying (Ouellet-Morin et al., 2012) indicate that epigenetic modifications can predispose individuals to risk or resilience over and above the variability induced by genetic factors, as found with maternal behavior in rodents. In fact, the sensitive period for establishing lasting effects of stress appears to extend beyond early life into juvenile and adolescent periods of development (McCormick and Mathews, 2010; Dudley et al., 2011), as evidenced by site-specific demethylation of *fkbp5* and reduced expression of DNMT1 in the mouse dentate gyrus of juvenile pups that were given 4 weeks of corticosterone treatment (Yang et al., 2012).

Although individual differences in PTSD risk are well established, relatively few studies have evaluated the specific mechanisms that underlie resilience. It is not yet clear whether resilience reflects the addition of a protective factor or the absence/reduction of a vulnerability factor (Russo et al., 2012). Studies of gene expression have found higher blood levels of NPY in resilient Special Forces soldiers (Morgan et al., 2000, 2002) and higher levels of c-Fos, FosB, and ΔFosB in resilient mice (Covington et al., 2010; Lehmann and Herkenham, 2011; Adamec et al., 2012), although genome-wide studies have reported altered expression in a comparable number of genes in resilient and vulnerable populations (Benatti et al., 2012). Some evidence suggests that risk and resilience are associated with opposite epigenetic modifications on similar genes, wherein resilience is associated with increased methylation and decreased expression of CRH and vulnerability is associated with demethylation and increased expression of CRH and *fkbp5* in response to chronic stress (Elliott et al., 2010).

Epigenetic writers themselves are also candidates for mediating risk and resilience, in part through managing an appropriate balance between epigenetic repressors and epigenetic activators (Zovkic et al., 2013). Under baseline conditions, epigenetic repressors, such as HDACs, keep gene expression in check, whereas neuronal signaling increases gene expression in part by relieving such inhibition (Lattal et al., 2007). This repressive action of HDACs and other molecules has been termed the molecular break pad, reflecting the involvement of these molecules in regulating the magnitude and the duration of gene expression (Lattal and Wood, 2013). For example, reduction of HDAC3 expression prolongs the expression of immediate early genes involved in learning and memory (McQuown and Wood, 2011; McQuown et al., 2011) and HDAC inhibition or knockdown enhances fear learning in rodents (Levenson et al., 2004; Guan et al., 2009; Graff and Tsai, 2011; Lesburgueres et al., 2011). An implication of these studies

is that a disrupted balance favoring activators over repressors may promote PTSD vulnerability by increasing the intensity of the traumatic memory, which is consistent with reports of reduced HDAC2 expression in the post-mortem brains of human patients suffering from depression (Sun et al., 2013).

However, not all HDAC subtypes are memory repressors. For example, sirtuins are class III HDACs that have been implicated in improved cognition (Kim et al., 2007) and hippocampal overexpression of HDAC1 was recently associated with improved extinction of fear memory (Bahari-Javan et al., 2012). Although sirtuins tend to be regulated by a distinct class of compounds, many pharmacological HDACi influence a broad range of HDACs, making it difficult to associate behavioral outcomes with specific HDAC subtypes. For example, although chronic social defeat was found to selectively increase the expression of the memory repressor HDAC2, treatment with the HDACi mass spectrometry (MS)-275 had an anti-depressant effect in these rodents (Guan et al., 2009; Covington et al., 2011b). This discrepancy may in part be associated with the relative specificity of MS-275 for HDAC1 over other HDACs, including HDAC2 (reviewed in Grayson and Guidotti, 2013). Similarly, TSA is an HDACi that actually increases HDAC1 expression in cell culture (Ajamian et al., 2004) and given the positive role of this HDAC on fear extinction (Bahari-Javan et al., 2012), TSA-induced enhancement of cognitive function may at least partly be a reflection of enhanced expression of HDAC1.

In addition to HDACs, DNMTs are emerging as another class of multifunctional epigenetic regulators with distinct modifications in PTSD, as evidenced by hypomethylation of DNMT3B gene and hypermethylation of the DNMT3L gene (Uddin et al., 2010). HDACs and DNMTs are only few of many epigenetic regulators involved in mediating the balance between activators and repressors and a thorough understanding of the potential shifts in their balance will require the consideration of these enzymes in the context of other epigenetic mediators, including histone acetylases, methylases, and demethylases, TET proteins, and other enzymes that directly mediate epigenetic modifications. Complementing these studies with genome-wide changes in gene expression will provide valuable information regarding the effects of specific modifications on genes associated with risk and resilience.

COGNITIVE ASPECTS OF PTSD: CAUSE AND OUTCOME

In addition to deregulation of the stress response, PTSD involves the development of strong cognitive associations between the stressful/fear-inducing event and the context or cue in which the event took place. This aspect is strongly tied into PTSD symptom expression, in that the stimuli or cues that are similar to those associated with trauma can on their own trigger re-experiencing of traumatic memories long after the event has passed (Ehlers et al., 2004). Epigenetic mechanisms are particularly relevant in this regard because of their role in the persistent stabilization of memory for transient fear-inducing stimuli (Zovkic et al., 2013). Existing literature on the epigenetics of fear learning has been conducted primarily in the context of cognition, but the use of fear conditioning models in PTSD makes these studies directly relevant to the disorder. Epigenetic modifications in fear learning have been extensively reviewed in a recent paper (Zovkic and Sweatt, 2013) and will be discussed here only briefly to illustrate the current state of understanding and the future directions required to advance the application of these models to mechanistic and clinical studies of PTSD.

A major advantage of animal models of fear learning is the ability to assess fear expression at defined time points after the fear-inducing stimulus to track the molecular mechanisms involved in the establishment and perpetuation of fear memory. Assessment of fear learning shortly after shock (2 h) best reflects the initial response to trauma, but this is only an index of short term memory and is not well suited for assessing persistent symptoms of PTSD, particularly given the lack of an initial emotional response in many patients with PTSD (Friedman et al., 2011a,b). However, this is a critical time point for evaluating epigenetic mechanisms of fear memory consolidation that are required for stabilizing the memory over time (Zovkic et al., 2013). Molecular assessment at this time point may reveal different patterns or intensities of gene expression and associated epigenetic modifications in vulnerable and resilient individuals that can inform efforts to identify mechanisms for pathological fear formation. Consolidation of cued and contextual fear conditioning is mediated by epigenetic modifications in the amygdala, whereas epigenetic modifications in the hippocampus are specifically involved in mediating contextual fear learning (Levenson et al., 2004; Lubin and Sweatt, 2007; Miller et al., 2008; Monsey et al., 2011). Consolidation of hippocampus-dependent fear memory is typically evaluated 24 h after training, whereas consolidation of remote memory becomes evident at least 7 days after training, when the memory from the hippocampus has been "downloaded" to the cortex for maintenance (Frankland et al., 2004; Ding et al., 2008). A focus on the remote time point is especially relevant because it provides the best approximation of the persistent and intrusive memories seen in patients with PTSD. Given the well-defined nature of the brain regions involved in fear consolidation and maintenance, the straight-forward behavioral read out of fear learning (i.e., freezing), and the ease with which stimulus (i.e., shock) intensity can be modified, this model provides an excellent platform on which to build our understanding of epigenetic mechanisms of PTSD. For a full review of epigenetic mechanisms of fear learning and their implications for PTSD, please refer to a recent review from our lab (Zovkic and Sweatt, 2013).

MEMORY CONSOLIDATION

It is now well established that epigenetic mechanisms are essential mediators of fear memory consolidation. Histone acetylation, phosphorylation, and methylation play a critical role in hippocampus- and amygdala-dependent consolidation across a range of conditioning paradigms (Chwang et al., 2006; Levenson et al., 2006; Lattal et al., 2007; Lubin and Sweatt, 2007; Bredy and Barad, 2008; Lubin et al., 2008; Miller et al., 2008; Maddox and Schafe, 2011; Monsey et al., 2011; Stafford et al., 2012) and interact with DNA methylation to mediate memory formation (Miller et al., 2008; Maddox and Schafe, 2011). Pharmacological or genetic interference with DNA methylation impairs fear memory (Miller and Sweatt, 2007; Miller et al., 2008, 2010; Feng et al., 2010), whereas enhancement of histone acetylation through HDAC inhibition enhances memory formation through reduced threshold for memory formation (Levenson et al., 2004; Bredy

and Barad, 2008; Miller et al., 2008; Graff and Tsai, 2011; Maddox and Schafe, 2011; Monsey et al., 2011). Similarly, knocking out *gadd45,* which is involved in DNA demethylation, improves memory performance (Sultan et al., 2012), indicating that memory may be enhanced through a paradoxical upregulation of both DNA methylation and histone acetylation. Although a number of explanations have been offered for this paradox (Zovkic et al., 2013), recent advances implicating DNA methylation in gene activation may also contribute to a resolution. Nevertheless, the observation of improved memory with HDACi indicates that individuals with lower levels of HDACs and higher levels of enzymes involved in DNA demethylation may be more sensitive to developing fear responses that are disproportional to the traumatic event. This proposition is particularly relevant in the context of the molecular breakpad hypothesis, in which synaptic activity overrides the normally repressive effects of HDACs on transcription (Lattal and Wood, 2013), in that lower levels of HDACs may allow subthreshold levels of synaptic activity to produce a strong memory. Thus, an essential aim of PTSD research must be to fully characterize the relationship between different epigenetic enzymes and PTSD, as they relate to the consolidation of traumatic experiences. Advances have already been made in studies of group differences under baseline conditions in studies of human patients, which found reduced levels of HDAC2 and altered methylation of DNMT3L genes in PTSD (Uddin et al., 2010; Sun et al., 2013).

MEMORY MAINTENANCE

Hippocampus-dependent memory consolidation occurs within hours of fear conditioning, when the memory begins a gradual process of downloading to the cortex for maintenance (Frankland and Bontempi, 2005). Epigenetic mechanisms have been implicated in the initial memory transfer process in which histone modifications in the cortex serve as a memory transfer tag (Lesburgueres et al., 2011) and as a persistent mark for memory maintenance (Graff et al., 2012). In contrast to the transient changes in DNA methylation observed in the hippocampus, fear conditioning is associated with a persistent increase in DNA methylation and decreased expression of the gene coding for the memory suppressor calcineurin up to 30 days after fear conditioning (Miller et al., 2010). DNA methylation is critical for memory maintenance at this time point, such that blocking DNA methylation immediately before a 30 day memory test impairs recall (Miller et al., 2010). The role of DNA methylation in memory persistence is of particular relevance to PTSD given the persistent re-experiencing of the traumatic event, indicating that manipulation of the epigenome at appropriate time points may allow for interference with previously established traumatic memories (see Zovkic and Sweatt, 2013 for an expanded discussion of therapeutic implications).

MEMORY RECONSOLIDATION AND EXTINCTION

Persistent and intrusive memory recall is the foremost feature of PTSD in humans (Orr et al., 1993). Studies of molecular events associated with recall are critical for understanding the molecular mechanisms that underlie the strength of the pathological memory and for identifying potential treatments. Memory recall is an active process that renders the memory labile and employs molecular mechanisms to re-stabilize the original memory (Bredy and Barad, 2008; McKenzie and Eichenbaum, 2011; Pitman et al., 2011; Lattal and Wood, 2013). From a treatment perspective, interference with reconsolidation provides an opportunity to destabilize and even erase the original traumatic memory and this avenue is being actively pursued in patients with PTSD (Pitman, 2011). A recalled memory also becomes subject to a second active process termed extinction. Extinction occurs when a previously learned association is altered by new information, such as the safety of a conditioned stimulus in which a shock had previously occurred (Bouton, 2004). In contrast to disrupted reconsolidation, which effectively promotes forgetting, enhancing extinction allows for new learning in which the fear-inducing component of the memory can be selectively disrupted while leaving the initial memory relatively intact (Lattal and Wood, 2013). PTSD is often considered to be a disorder of extinction (Koenigs and Grafman, 2009) and many studies with rodents have found impaired extinction in at-risk individuals. For example, low LG and maternally separated offspring exhibit enhanced fear conditioning and impaired extinction (Champagne et al., 2008; Bagot et al., 2009; Callaghan and Richardson, 2011), whereas others associated brief maternal separation (15 min) with impaired fear extinction in spite of similar rates of initial conditioning (Wilber et al., 2007, 2009; Stevenson et al., 2009). Deficits in extinction have also been associated with the serotonin transporter in rodents (Wellman et al., 2007; Narayanan et al., 2011) and in people (Hartley et al., 2012), indicating that genetic risk for PTSD may be partly mediated by impaired fear extinction.

A major challenge in applying extinction and reconsolidation as a long-term treatment is the relative transience of extinction relative to the original learning event and the requirement for pharmacological intervention for robust interference with reconsolidation (Pitman, 2011). However, recent studies in rodents suggest that extinction may be an effective treatment strategy when combined with manipulations of the epigenome that can stabilize extinction learning over time (Lattal et al., 2007; Bredy and Barad, 2008; Lattal and Wood, 2013). Specifically, administration of HDACi promotes extinction of fear learning and robust extinction of cocaine preference that is resistant to subsequent reinstatement by cocaine (Malvaez et al., 2010), indicating that HDAC inhibition combined with extinction may promote adaptive learning that can combat deleterious adaptations. The effects of HDACi on extinction are dependent primarily on the HDAC1 subtype (Bahari-Javan et al., 2012) and are most effective in the infralimbic cortex (Stafford et al., 2012), which is consistent with evidence that HATs mediate extinction specifically in this brain region (Marek et al., 2011; Wei et al., 2012). This regional specificity is of functional relevance because it selectively alters extinction without impacting initial learning (Wei et al., 2012) and of translational relevance because lesions of the ventromedial prefrontal cortex (vmPFC) impair long-term extinction in people (Quirk et al., 2000) and reduced vmPFC activity has been reported in patients with PTSD (Koenigs and Grafman, 2009). These studies point to a dissociation between fear learning and fear extinction, wherein deficits in extinction are a critical characteristic of PTSD (Schnurr and Friedman, 2008) and may be more relevant to treatment because of the temporal gap between trauma and treatment onset. This temporal and regional difference also has implications

for HDAC treatment, in that HDAC inhibition around the time of trauma exposure increases fear memory, whereas HDAC inhibition in association with fear extinction enhances the extinction of fear memory. This distinction is important to keep in mind when developing therapies for PTSD.

Extinction, in the form of exposure therapy, is heavily utilized treating PTSD (Schnurr and Friedman, 2008) and some effort has been made toward implementing reconsolidation based approaches in treatment (Pitman, 2011). However, more work is needed to identify the best conditions under which behavioral therapies are most effective when combined with pharmacological therapies. Clinical studies suggest that contextual parameters utilized in extinction and reconsolidation based therapies are critical for treatment efficacy, in that similarity between treatment conditions and those associated with the initial trauma has a major impact on treatment outcomes (Pitman, 2011). In humans and in animal models, this concept is typically investigated under the umbrella of fear generalization, whereby neutral cues can elicit fear responses based on their degree of similarity to cues or contexts associated with traumatic stimuli (Dunsmoor et al., 2009; Iordanova and Honey, 2012), but we are not aware of studies that have directly investigated epigenetic mechanisms in relation to fear generalization. In humans, there is increased use of virtual reality-based therapies to better reconstruct traumatic scenarios (Davis et al., 2006a,b) and similar approaches can be used to predict traumatic scenarios for soldiers before going into combat. Nevertheless, it is still necessary for animal studies to combine a thorough behavioral assessment with epigenetically based pharmacological interventions to identify the appropriate conditions for subsequent testing in human patients.

EPIGENETICS IN DIAGNOSIS AND INTERVENTION

Epigenetic modifications may be particularly strong candidates for use in assessing at-risk individuals for early intervention, as well as for PTSD treatment through epigenetically based therapies. An important first step toward such use of epigenetics involves the establishment of "epigenetic signatures" that serve as diagnostic markers and help identify targets for directed treatment. The idea of an epigenetic signature is driven by the assumption that PTSD is associated with consistent alterations in the epigenome across individuals and that these modifications can be used as markers of risk, resilience, and symptom severity. We have discussed much of the substantial progress that has been made toward identifying common epigenetic modifications in at-risk and resilient populations (see discussion of gene-environment-epigenome interactions above) and systematic investigations of these modifications can be of tremendous importance for directing early intervention (Uddin et al., 2010). Indeed, studies in animal models have exhibited encouraging results regarding the reversal of risk factors and cognitive deficits through enrichment and epigenetic intervention in juvenile and adult rodents (e.g., Francis et al., 2002; Fischer et al., 2007), indicating that early detection of risk may be key for PTSD prevention. Epigenetic markers will also be useful in PTSD diagnosis and in tracking therapeutic efficacy after trauma.

Although it is unlikely that any two individuals will have identical epigenetic profiles in response to trauma, it is conceivable that a substantial amount of overlap in essential genes will be found across patients, as evidenced by reports of altered *DNMT*, *FKBP5*, *SLC6A4*, and *GR* methylation reviewed above. Moreover, recent studies have demonstrated similar levels of DNA methylation of *SLC6A4* and *FKBP5* genes in the blood and the brain (Suomi, 2011; Klengel et al., 2013), indicating that the epigenetic status of certain genes in the brain may be indicated by changes in the blood. However, differences in function between immune cells and neurons must be taken into account, as early life stressors are known to modify immune cell function (Lam et al., 2012) and immune system alterations are commonly observed in PTSD (Smith et al., 2011). The degree to which concordance between brain and blood is replicable and applicable to other genes remains to be determined, but such concordance is less relevant for many clinical purposes than the ability of peripheral markers to reliably predict PTSD symptoms.

Nevertheless, tracking changes in pre- and post-treatment epigenetic profiles would be particularly useful for identifying genes that are modified by behavioral and epigenetic interventions as a first step in developing targeted therapies. In addition, it may be possible to identify the changes that are associated with persistent positive outcomes and to use this information to tailor more effective therapies. Indeed, an extinction-specific role of HDAC1 has already been identified for fear learning and a library of such markers would provide a valuable tool for designing epigenetic therapies to specifically enhance the efficacy of corresponding behavioral therapies. The efficacy of epigenetic intervention is evidenced by the fact that anti-depressant drugs produce epigenetic modifications (Tsankova et al., 2004, 2006) and that epigenetic drugs have anti-depressant effects (Covington et al., 2011b).

Epigenetic therapies, including HDACi, may be particularly strong candidates for PTSD treatment because they do not target a single neurotransmitter system and often exhibit therapeutic or behavioral effects only under appropriate signaling conditions (Roozendaal et al., 2010; Graff and Tsai, 2011). These pharmacological features are relevant to many psychiatric disorders, including PTSD, which exhibit deregulation of multiple neurotransmitter and signaling molecules (e.g., serotonin, NPY, GR, Fkpb5, BDNF, and CRH) and are typically treated by drugs such as fluoxetine that are targeted against specific neurotransmitter systems (Steckler and Risbrough, 2012). It is now evident that up- and down-regulation of the various deregulated molecules is at least partly mediated by altered DNA methylation and histone acetylation (Costa et al., 2003; Dong et al., 2007; Kinnally et al., 2011; Koenen et al., 2011; Klengel et al., 2013), indicating that epigenetic drugs may stabilize function across a range of disrupted molecules. For example, upregulation of *FKBP5* in high-risk individuals is associated with reduced methylation of the GRE, which is demethylated by exposure to glucocorticoids during early development (Klengel et al., 2013). Similarly, reduced levels of reelin and 67-kDA glutamate decarboxylase (GAD_{67}) have been attributed to DNA hypermethylation in mice (Costa et al., 2003; Dong et al., 2007).

On the flip side, many of the molecules we are discussing also alter gene expression through downstream effects on the epigenome and some evidence suggests that the bidirectional relationship across levels of regulation is a key driver or appropriate and stimulus-specific effects on behavior. For example, PKA-pCREB/CBP signaling and GR receptor activation drive changes in histone acetylation and hippocampus-dependent learning (Levenson et al., 2004; Roozendaal et al., 2010; Maddox and Schafe, 2011; Monsey et al., 2011), but HDACi could only enhance learning in the presence of GR activation (Roozendaal et al., 2010), indicating that increasing histone acetylation exerts behavioral effects only in the presence of appropriate signaling.

In addition to driving gene expression in mental disorders, epigenetic drugs may also normalize deregulated gene expression. For example, reduced gene expression and increased DNA methylation in schizophrenia were reversed by treatment with HDACi and this effect was associated with an improvement in psychotic symptoms (Costa et al., 2003; Tremolizzo et al., 2005a; Dong et al., 2007). Similarly, positive outcomes were reported for the HDACi valproic acid when given in conjunction with antipsychotics (Wassef et al., 2000, 2001; Grayson et al., 2010) or anti-depressants (Schroeder et al., 2007). Additionally, studies in rodents reported enhanced extinction of fear (Lattal et al., 2007; Bredy and Barad, 2008) and reduced depression (Covington et al., 2011b) in response to HDACi treatment, indicating that these drugs may be efficacious through effects on cognitive and affective processes. Even treatments that do not directly manipulate the epigenome produce epigenetic changes, with behavioral enrichment improving cognitive deficits through similar effects on chromatin regulation as HDACi (Fischer et al., 2007). Thus, a combination of environmental and pharmacological treatments may provide strongest candidates for early intervention or treatment. However, it is important to caution that outcomes associated with HDAC treatment in humans have been mixed (Narayan and Dragunow, 2010) and may be partly attributed to administration of adjunct therapies and the stage of the disease at the time of treatment (Tsai review). The development of more specific drugs and under appropriate conditions will be essential for improving the clinical efficacy of epigenetic therapies.

A number of issues must be addressed in designing epigenetic drugs with greater efficacy, including improved blood-brain-barrier (BBB) permeability and increased specificity for brain regions and HDAC subtypes (Grayson et al., 2010). HDACs are differentially distributed throughout the brain (Broide et al., 2007) and a growing number of studies point to distinct effects of specific HDAC subtypes in distinct brain regions and behavioral phenomena (Kim et al., 2007; Bahari-Javan et al., 2012; Sun et al., 2013). There is already a range of drugs that have different levels of regional and subtype specificity, with one study reporting that systemic injections of the BBB-permeable MS-275 (an HDACi selective for HDAC1 over HDAC3 and 8) selectively enhanced H3 acetylation in the frontal cortex and the hippocampus, but not the striatum (Simonini et al., 2006). In that study, MS-275 was more potent than valproic acid, which induced similar changes in acetylation across brain regions (Simonini et al., 2006). With more studies demonstrating HDAC-subtype specific effects on neural function and behavior (Kim et al., 2007; Bahari-Javan et al., 2012; Hanson et al., 2013), drugs that can be systemically administered and produce selective effects will be critical for targeted treatments. Interestingly, studies of human T23 bladder and MDA breast carcinoma, as well as HL60 cells, found a similar proportion of genes to be up- and down-regulated in response to treatment with HDACi (Glaser et al., 2003; Halsall et al., 2012), indicating that the effects of HDACi are not exclusively activating. Indeed, a high level of complexity and balance between memory activators and repressors necessitates that some genes are turned off in order for others to be effectively turned on (Zovkic et al., 2013).

Discussions of epigenetic treatments rightfully caution about the potential for wide-spread and non-specific effects on gene expression. However, microarray analysis of changes in hippocampal gene expression in response to l-methionine treatment only affected 1% of all the genes (Weaver et al., 2006), indicating that manipulations of epigenetic precursors and enzymes may produce surprisingly specific outcomes. Similarly, treatment of HL60 cells with three commonly used HDACi (VPA, TSA, SAHA) altered the expression of approximately 9% of genes, with only minor increases in histone acetylation observed at gene promoters (Halsall et al., 2012).

Moreover, manipulations of HDACs result in at least partly discriminant changes in acetylation, with HDAC1 having no effect on AcH3K14 and AcH4K5 (Bahari-Javan et al., 2012) and MS-275 altering AcH3K9 without affecting H4K12 (Simonini et al., 2006; Peleg et al., 2010). In addition to gene- and residue-specific effects, HDAC2 selectively enhances inhibitory synaptic function in the CA1 (Hanson et al., 2013), indicating that there is potential for specificity among epigenetic therapies. Much work is still needed to improve target specificity and the mechanisms of HDACi actions are not always clear, given the potential for the disruption of repressive complexes and effects on non-histone targets, which can be difficult to tease apart from effects of these drugs on histone acetylation (Zovkic et al., 2013). Thus, the development of increasingly specific therapies and a full characterization of the effects of existing therapies are important for developing drugs that can influence specific types of neural activity in selected brain regions.

FUTURE DIRECTIONS

In the present review, we focused heavily on developmental and genetic factors that predispose or protect individuals from PTSD. In many of these studies, the degree to which a manipulation is adaptive in adulthood is determined by reactivity to acute stressors outside of the context of PTSD. While these studies provide valuable insights into variable responses to trauma, a thorough assessment of persistent symptoms over prolonged periods of time is required to better model the lasting and intrusive nature of PTSD in people. Fear conditioning is one example of a model that is amenable to testing at different time points that can be used to characterize epigenetic changes that extend beyond the initial response to trauma and include trauma maintenance (see **Tables 1** and **2** for summary of epigenetic modifications in fear conditioning and PTSD models). It will also be important to combine developmental models with models of PTSD that sort animals according to symptom development, severity, and persistence. This

Table 1 | A summary of epigenetic modifications reported in rodent models of fear conditioning.

Epigenetic modification measured	Gene	Brain region	Effect	Reference
MEMORY CONSOLIDATION (30 min–2 h AFTER FEAR CONDITIONING)				
H3 acetylation	Global	CA1	↑	Chwang et al. (2006), Levenson et al. (2004), Miller et al. (2008)
	Bdnf IV promoter	CA1	↑	Lubin et al. (2008)
		Hippocampus	↑	Takei et al. (2011)
	Homer 1 promoter	Hippocampus	↑	Mahan et al. (2012)
	Global	Lateral amygdala	↑	Monsey et al. (2011), Maddox et al. (2013)
H3 phosphorylation	Global	CA1	↑	Chwang et al. (2006)
H3 phosphoacetylation	Global	CA1	↑	Chwang et al. (2006)
H3K9me2	Global	Entorhinal cortex	↑	Gupta-Agarwal et al. (2012)
H3K4me3	Global	CA1	↑	Gupta et al. (2010), Gupta-Agarwal et al. (2012)
	zif268 promoter	CA1	↑	Gupta et al. (2010)
	BDNF I	CA1	↑	Gupta et al. (2010)
	Homer 1 promoter	Amygdala	↓	Mahan et al. (2012)
DNA methylation	PP1	CA1	↑	Miller and Sweatt (2007)
	Reelin		↓	Miller and Sweatt (2007)
	Bdnf		↓	Lubin et al. (2008)
	zif268		↑	Gupta et al. (2010)
MEMORY MAINTENANCE (7–30 DAYS)				
DNA methylation	Calcineurin	PFC	↑	Miller et al. (2010)

will be especially useful in distinguishing between early life experiences acting as stress inoculators vs. risk inducers in relation to adult risk and resilience. Ideally, early life manipulations will be combined with genetic models to reflect the gene-environment interactions identified in human risk populations. It is important to note that the rapid pace at which novel techniques are developed poses an opportunity and a challenge for conducting cutting edge research that can shed new light on our understanding of PTSD and psychiatric disorders. Most rapid advancements are particularly evident for techniques differentiating between DNA methylation and hydroxymethylation. Given the role of 5hmC in DNA demethylation and the distinct association of each modification with gene regulation, we have included a summary of new approaches to facilitate their application in studies of PTSD (see **Box 1**).

In addition to affecting gene expression through the direct interaction with transcriptional machinery, DNA methylation is increasingly being recognized as an incredibly complex regulator of neuronal function and metaplasticity (Baker-Andresen et al., 2013). For example, *de novo* DNA methylation was shown to prevent RNA polymerase stalling, thus providing a mechanism by which DNA methylation can regulate alternative splicing (Shukla et al., 2011). The long-term regulation of alternative splicing by DNA methylation may affect the responsivity of neurons to future stimuli (Baker-Andresen et al., 2013); thus investigations into the epigenetic regulation of alternative splicing are warranted in delineating factors that convey resilience to PTSD. Additionally, DNA methylation has been implicated in the regulation of retrotransposons, such as the positioning of long interspersed nuclear elements-1 (LINE-1) (Muotri et al., 2010). This is important, as the insertion of LINE-1 into various genomic regions may increase gene length, which is associated with decreased efficiency of expression (Castillo-Davis et al., 2002). Interestingly, a recent report demonstrated that a post-deployment diagnosis of PTSD was associated with hypomethylation of LINE-1, suggesting that methylation of these elements may play a role in mediated risk or resilience (Rusiecki et al., 2012). The existence of 5hmc and of distinct cofactors that interact with 5mc and 5hmc (Spruijt et al., 2013) further complicates the interpretation and calls for the use of techniques that distinguish between these modifications (see **Box 1**). Such approaches will be critical for identifying epigenetic signatures that differentiate between risk and resilience.

Finally, although women are more likely to develop PTSD than men, mot rodent studies have been conducted only in males (Mulchahey et al., 2001; Pope et al., 2003). Thus, a greater focus on females is warranted and may help us understand the basis for relative resilience in men, with testosterone providing one possible candidate for resilience (Russo et al., 2012). Some existing evidence suggests that sex differences are will be relevant for understanding all aspects of PTSD, with prenatal stress producing altered levels of DNMT1 expression, hippocampal GR methylation, and hypothalamic CRH methylation in adult male, but not female, offspring (Mueller and Bale, 2008). In addition, evidence from rodent studies suggests that sex differences vary for distinct components of PTSD, with females exhibiting vulnerability in tests of motivation and affect, while exhibiting resilience in non-stressful cognitive tasks (Luine, 2002).

CONCLUSION

In this review, we have demonstrated that epigenetic mechanisms are fundamental regulators of predisposition and resilience to PTSD, primarily through mediating gene-environment interactions during sensitive periods of development. Epigenetic mechanisms are also essential mediators of proximal causes of PTSD

Table 2 | A summary of epigenetic modifications in human and animal models of PTSD.

Species/model	Gene(s) of interest	Major findings	Reference
CANDIDATE-GENE STUDIES			
Human	*ADCYAP1*, *ADCYAP1R1*	PTSD symptoms correlated with *Adcyap1r1* locus in women	Ressler et al. (2011)
Rat – predator odor + social instability	*Bdnf*	↑ Exon IV methylation in dorsal DG and CA1, ↓ exon IV methylation in ventral CA3, ↓ exon IV mRNA in both dorsal and ventral CA1	Roth et al. (2011)
Human	*SLC6A4*	Controlling for genotype, *SLC6A4* methylation modified the effect of PTEs on PTSD: ↓ *SLC6A4* promoter methylation associated with ↑ PTSD risk; ↑ *SLC6A4* promoter methylation was protective against PTSD	Koenen et al. (2011)
Human	*SLC6A3*	↑ *SLC6A3* promoter methylation associated with ↑ risk of lifetime PTSD in 9R allele carriers	Chang et al. (2012)
Human	*COMT*	*COMT* Met/Met genotype interacted with CpG methylation in mediating impaired fear inhibition in PTSD patients	Norrholm et al. (2013)
Human	*FKBP5*	GC exposure was associated with ↑ *FKBP5* GRE demethylation and ↑ *FKBP5* expression in carriers of the risk compared with the protective allele	Klengel et al. (2013)
GENOME-WIDE/LARGE SCALE STUDIES			
Human	Genes involved in immunity, neurogenesis, the startle response, *DNMT3B*, *DNMT3L*, imprinted genes: *NDN, MAGEL2, ATP10A*	PTSD was associated with: (1) ↑ methylation of *DNMT3B*, ↓methylation of *DNMT3L*; (2) degulated methylation of genes involved in Prader–Willi and Angelman syndromes; (3) methylation profiles suggesting upregulation of immune-related genes and downregulation of genes involved in neurogenesis and the startle response	Uddin et al. (2010)
Rat – predator odor	*Dlgap2*, *Dll3*, *Pkcη*, *Rps6kb2*	Of the four differentially methylated genes identified, *Dlgap2* was associated with a change in mRNA expression. ↓ intragenic methylation associated with ↓ hippocampal mRNA expression	Chertkow-Deutsher et al. (2010)
Human	*TPR*, *CLEC9A*, *APC5*, *ANXA2*, *TLR8*, *BDNF*, *CXCL1*, immune-related genes	PTSD associated with: (1) ↓ methylation of *TPR* and *ANXA2* and ↑ methylation of *CLEC9A*, *APC5*, *TLR8* in PTSD; (2) ↑ methylation of BDNF and CXCL1; (3) 19 of 54 of the differentially methylated immune-related genes examined in Uddin et al. (2010)	Smith et al. (2011)
OTHER			
Human	33 loci previously associated with PTSD	Only *MAN2C1* showed evidence of interaction with PTE no. in PTSD risk: ↑ *MAN2C1* methylation interacted with ↑ no. of PTEs to ↑ PTSD risk	Uddin et al. (2011)
Human	Repetitive elements: *LINE-1*, *Alu*	In US military service members recently deployed to Afghanistan or Iraq: LINE-1 was hypomethylated in PTSD cases vs. control post-deployment. *Alu* was hypermethylated in PTSD cases vs. control pre-deployment	Rusiecki et al. (2012)

PTE, potentially traumatic event; TLS, total life stress; ADCYAP1, adenylate cyclase-activating polypeptide 1 (pituitary); ADCYAP1R1, adenylate cyclase-activating polypeptide 1 (pituitary) receptor type I; Bdnf, brain-derived neurotrophic factor; SLC6A4, solute carrier family 6 (neurotransmitter transporter, serotonin), member; SLC6A3, solute carrier family 6 (neurotransmitter transporter, dopamine), member; COMT, catechol-O-methyltransferase; FKBP5, FK506 binding protein 5; DNMT3B, DNA methyltransferase-3B; DNMT3L, DNA methyltransferase-3L; NDN, necdin, melanoma antigen (MAGE) family member; MAGEL2, MAGE-like 2; ATP10A, ATPase, class V, type 10A; Dlgap2, disks large-associated protein 2; Dll3, delta-like 3; Pkcη, protein kinase C η; Rps6kb2, ribosomal protein S6 kinase polypeptide 2; TPR, translocated promoter region; CLEC9A, C-type lectin domain family 9, member A; APC5, acid phosphatase 5, tartrate resistant; ANXA2, annexin A2; TLR8, toll-like receptor 8; CXCL1, chemokine (C-X-C motif) ligand 1; MAN2C1, mannosidase, alpha, class 2c, member 1.

because of their role in stabilizing persistent outcomes of transient traumatic events. Although much progress has been made toward identifying epigenetic contributions to PTSD, ongoing shifts in our fundamental understanding of epigenetic modifications and their function call for the use of new technologies to clarify the role of these modifications in psychiatric disorders. As our appreciation of the complex roles of epigenetic mechanisms increases, so will our ability to create new and effective interventions for this incredibly complex and devastating human disease.

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Making memories of stressful events: a journey along epigenetic, gene transcription, and signaling pathways

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Strong psychologically stressful events are known to have a long-lasting impact on behavior. The consolidation of such, largely adaptive, behavioral responses to stressful events involves changes in gene expression in limbic brain regions such as the hippocampus and amygdala. However, the underlying molecular mechanisms were until recently unresolved. More than a decade ago, we started to investigate the role of glucocorticoid hormones in signaling and epigenetic mechanisms participating in the effects of stress on gene transcription in hippocampal neurons. We discovered a novel, rapid non-genomic mechanism in which glucocorticoids via glucocorticoid receptors facilitate signaling of the ERK-MAPK signaling pathway to the downstream nuclear kinases MSK1 and Elk-1 in dentate gyrus granule neurons. Activation of this signaling pathway results in serine10 (S10) phosphorylation and lysine14 (K14) acetylation at histone H3 (H3S10p-K14ac), leading to the induction of the immediate-early genes c-Fos and Egr-1. In addition, we found a role of the DNA methylation status of gene promoters. A series of studies showed that these molecular mechanisms play a critical role in the long-lasting consolidation of behavioral responses in the forced swim test and Morris water maze. Furthermore, an important role of GABA was found in controlling the epigenetic and gene transcriptional responses to psychological stress. Thus, psychologically stressful events evoke a long-term impact on behavior through changes in hippocampal function brought about by distinct glutamatergic and glucocorticoid-driven changes in epigenetic regulation of gene transcription, which are modulated by (local) GABAergic interneurons and limbic afferent inputs. These epigenetic processes may play an important role in the etiology of stress-related mental disorders such as major depressive and anxiety disorders like post-traumatic stress disorder.

Keywords: stress, glucocorticoid hormone, epigenetic, MAPK, immediate-early gene, learning and memory, PTSD, anxiety

INTRODUCTION

The mnemonic function of the brain is one of its most important cognitive attributes. Making memories of events in our lives is vital in order to find, also quite literally, our way around in life. The formation of memories allows us to interact spatially, socially, and otherwise with our environment. As memories are built on representations from the environment, they are a pivotal part of how we adapt to changes in the environment thereby preparing us cognitively, emotionally, and physiologically should a similar situation occur in the future. We make particularly strong memories of traumatically stressful events in our lives. Most people cope well with such ordeals and stay healthy suggesting that they have adapted successfully. Some people however develop an anxiety disorder like post-traumatic stress disorder (PTSD), which seriously compromises their quality of life for a long time, often life-long. They are burdened by nightmares, reliving the incident recurrently, negative associations, mood swings, strong vegetative characteristics, and other debilitating symptoms. Clearly, strong memories have been encoded in these individuals but adaptation and coping mechanisms have failed. Until now, the question why 10–20% of the population develops a stress-related disorder after experiencing a traumatic, often life-threatening experience has remained unanswered. In order to answer this question, we need to obtain insight into how stress interacts with the emotional and cognitive processing of an event at the molecular level in the brain. Obtaining this knowledge is fundamental to answering the question about the neurobiological basis underlying the vulnerability for developing a stress-related mental disorder.

ANIMAL MODELS

The investigation of the effects of stress on learning and memory processes in the brain requires the use of animal models. The brain region which has received most attention is the hippocampus, a limbic brain structure, which is vital for the consolidation of contextual memories and plays a major role in coordinating the behavioral, autonomic, and neuroendocrine responses to stress. It has been shown in a number of studies that stress-induced glucocorticoid hormones (corticosterone in rats and mice) enhance the consolidation of memory formation in various hippocampus-dependent behavioral models including the contextual fear conditioning paradigm, the Morris water maze, and forced swimming-induced behavioral immobility (1–5). In fact, stress is inherent to these behavioral tests as a rodent is not keen on receiving an electric shock or being put in a water basin, this inadvertently leading to an

enhanced secretion of glucocorticoid hormone from the adrenal gland (6).

An animal model often studied with regard to the role of glucocorticoid hormones in memory consolidation is the forced swim test. The strict dependency of the behavioral immobility response of glucocorticoid receptor (GR)-occupying levels of glucocorticoid hormone has been reported independently by two research groups in Utrecht, The Netherlands, and Melbourne, Australia (3, 5). The forced swim test is a rather straightforward test consisting basically of an (initial) test and a re-test. A rat or a mouse is put in a container (diameter < 30 cm) filled with water, usually 25°C, in which it cannot stand and from which it cannot escape. Usually, rats are left in for 15 min, mice often 10 min or shorter. After initial attempts of trying to escape by struggling or climbing movements vertically along the wall and horizontal swimming movements, the animal will acquire an immobile or floating posture. In the forced swim test's classic design, the animals are re-introduced to the water container for 5 min, 24 h after the initial test (7, 8). In this 5-min re-test, the animals struggle or swim relatively little but show mainly (70–75% of the 5-min time-period) immobility/floating behavior. Recently, we reported that animals also show this enhanced immobility behavior when tested 4 weeks after the initial forced swim test (9). The rodent displays this behavioral immobility response in the re-test because it has learnt from the previous experience that attempting to escape is futile and thus conserving energy by floating or immobility behavior is the best strategy for survival (10–15). Moreover, the animal may remember that it was taken from the water at the time of the first test. Thus, the enhanced behavioral immobility behavior displayed in the re-test is an adaptive response, which is based on memories formed of the initial forced swim experience (13–15). The extent of the immobility behavior displayed depends on the conditions under which the test is conducted: in warmer water (e.g., 35°C), rats show more immobility behavior whereas they show less of this behavior in cold water (19°C) (16). Under the latter conditions, presumably the animals struggle and swim more to combat the vast body temperature loss (approximately 12°C within 15 min) due to the cold water (17). These observations underline that the animals adapt their behavior in response to the context of the test (see **Figure 1**).

The forced swim test has been often used as a psychopharmacological test for antidepressant drug screening. Acute treatment (up to three injections) of rats or mice with several (e.g., TCAs, SSRIs) but not all antidepressant drugs typically result in decreased immobility behavior and more struggling and swimming behavior in the test and the re-test (8). Notably, acute treatment with these drugs evokes increased extracellular concentrations of neurotransmitters like serotonin, noradrenalin, and/or dopamine in many forebrain structures; i.e., neurochemical changes, which under physiological conditions are associated with enhanced motor activity and arousal mechanisms. Accordingly, the increased struggling/swimming behavior observed after antidepressant drug administration may be explained by a disruption of the immobility behavior the animals would normally display in this test. Therefore, using the forced swim test as a pharmacological test for screening new drugs for their potential

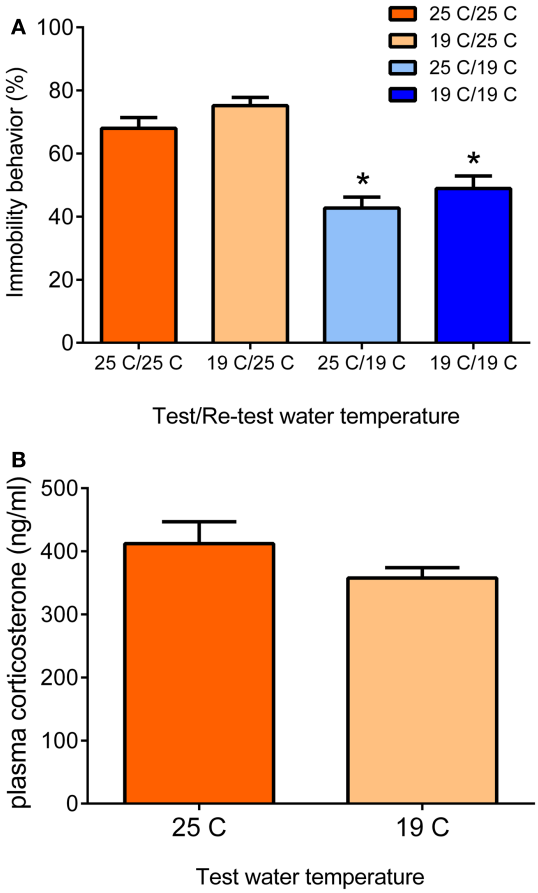

FIGURE 1 | Behavioral immobility response in the re-test depends on the water temperature during the re-test (A). Male Wistar rats were forced to swim for 15 min in 19 or 25°C-water (test). Twenty four hours later, they were forced to swim again for 5 min in 19 or 25°C-water (re-test). The data are presented as mean percentage immobility behavior during the 5-min re-test procedure ± standard error of the mean (SEM; $n = 11$–15) of different test/re-test water temperature conditions (see *x*-axis and legend). The data show that the immobility response in the re-test depends on the water temperature during the re-test and is independent of the water temperature at the time of the initial 15-min test. At 25°C re-test water temperature, the rats present the typical immobility response irrespective of the water temperature during the initial test. At 19°C re-test water temperature, the animals show significantly lower immobility scores, also irrespective of the water temperature during the initial test. Thus, in the Re-test, rats choose a behavioral strategy to cope with a challenge depending on conditions at the time of the challenge. The differential behavioral responses were not due to different responses in plasma glucocorticoid hormone **(B)**. Male Wistar rats were forced to swim for 15 min in 19 or 25°C-water and killed at 30 min after start of the forced swim procedure. Data are expressed as mean plasma hormone levels ± SEM (nanogram/milliliter; $n = 9$). Statistical analysis: **(A)** Two-way ANOVA: effect of test water temperature: $F(1,49) = 3.718$, $P = 0.06$ [Thus, there was a trend that if rats had swum at 19°C in the test, they would show a slightly higher immobility response in the re-test (see also Ref. (18)]. Effect of re-test water temperature: $F(1,49) = 55.163$, $P < 0.0005$. The interaction term was not statistically significant. **(B)** Student's *t*-test: $P > 0.05$, not significant. Johannes M. H. M. Reul and Sabine Ulbricht conducted this study at the Max Planck Institute of Psychiatry in Munich, Germany, unpublished data.

"antidepressant" activity has its limitations. Furthermore, it should be emphasized that this is a test for potential antidepressant drug action, not for depression. Hence, denoting rats or mice showing less struggling/swimming and more immobility behavior as being "depressed," is inappropriate.

As mentioned, the behavioral immobility response observed in the re-test is critically dependent of glucocorticoid hormone action during or shortly after the initial test. Thus, glucocorticoids are needed for the acquisition and consolidation of memories associated with the stressful challenge (9, 18, 19). Behavioral responses in the re-test are impaired in adrenalectomized (ADX) rats, which can be rescued by administration of corticosterone or the synthetic glucocorticoids, dexamethasone and RU28362, but not by administration of the mineralocorticoid hormone aldosterone or the progestin progesterone (3, 5). Thus, of the two glucocorticoid-binding receptors in the brain, i.e., the mineralocorticoid receptor (MR) and the GR (20–22), the latter receptor type appeared to be the participating one. Furthermore, impairment of immobility behavior in the re-test (but not in the initial test) was observed if rats were pre-treated with the GR antagonist RU38486 but not with the MR antagonists, spironolactone or RU28318 (23, 24). As RU38486 also has anti-progestin activity, the role of progestins was further clarified. It was found that the effect of RU38486 on immobility behavior could be rescued with dexamethasone but not with the synthetic progestin, R5020 (promegestone) (23). To obtain insight into the identity of the neuroanatomical substrate of the GR-mediated glucocorticoid effect on the behavioral immobility response, De Kloet et al. infused RU38486 into the dentate gyrus (DG), parafascicular nucleus, or paraventricular nucleus of the hypothalamus before the initial swim test (23). They found a significantly impaired behavioral immobility response if the drug had been infused into the DG but not into any of the other nuclei (23). These findings indicated that GRs in the DG are particularly important for the consolidation of this behavioral response.

EARLY EPIGENETIC OBSERVATIONS

The research pointing to a major role of glucocorticoid hormones in the forced swim test and other hippocampus-dependent tests was mainly conducted in the 1980s and early 1990s. For many years, it remained unclear how glucocorticoids are affecting these hippocampus-dependent behaviors. However, by the end of the 1990s, we made a serendipitous discovery: we found sparsely distributed granule neurons in the DG, which showed a speckled nuclear staining pattern for the chromatin-associated protein histone H3 phosphorylated at Serine10 (S10) and acetylated at Lysine14 (K14) (18). Nowadays, the code for this dual histone mark is H3S10p-K14ac. It turned out that under baseline conditions the number of immuno-positive neurons was very low but they increased considerably after psychologically stressful situations (known to be processed by the hippocampus) such as forced swimming, novelty, predator exposure, Morris water maze training, and fear conditioning (18, 25). Exposure to a cold environment or ether vapor, i.e., physical (non-hippocampal) stressors, was ineffective (18). Furthermore, we made an interesting observation that treating rats with the GR antagonists RU38486 or ORG34517 strongly inhibited the forced swimming-induced increase in H3S10p-K14ac-stained neurons in the DG (9, 18). ORG34517 is a rather novel GR antagonist, which has much less progesterone receptor antagonistic properties than RU38486 (26, 27). Based on *in vitro* work, it had been proposed that these epigenetic histone marks were involved in the opening of the chromatin structure rendering the hitherto silent genes located within this part of the chromatin accessible for transcription factors and other transcription-associated protein complexes and hence available for gene transcription (28–30). Thus, just after the turn of the millenium, our research had found evidence for stress- and glucocorticoid-sensitive histone modifications in DG neurons, which appeared to be related to transcriptional activation; a potentially interesting phenomenon but at the time not more than that. Moreover, at the time it was unclear whether this phenomenon had any bearing on the mechanisms underlying the behavioral immobility response.

Subsequent *in vitro* work of Mahadevan and colleagues in Oxford, UK, showed that the H3S10p-K14ac histone marks are associated with promoters of immediate-early genes (IEGs) like *fos* and *egr1* upon gene induction (31). We indeed found sparsely distributed c-Fos and Egr-1 immuno-positive neurons in DG of rats and mice, which increased in numbers after exposure to psychological stressors such forced swimming, novelty, and Morris water maze training (9, 18, 25, 32) (Carter et al., unpublished observation). Double immuno-fluorescence studies provided evidence that H3S10p-K14ac and c-Fos and Egr-1 protein co-localize in DG granule neurons (9, 25). Furthermore, pre-treatment of rats with the GR antagonist not only inhibited the forced swimming-evoked increase in H3S10p-K14ac in DG neurons but also strongly inhibited the stress effect on c-Fos and Egr-1 (9). Ultimate proof for a "physical" link between the H3S10p-K14 histone marks and c-Fos/Egr-1 was delivered by recent chromatin immunoprecipitation (ChIP) studies, which showed that these dual histone marks are present within the *fos* and *egr1* gene promoters of rats after forced swimming (9). Presently, ChIP studies on the H3S10p-K14ac and other histone marks in combination with next-generation Illumina sequencing are underway to make a genome-wide assessment of all genes associated with specific histone marks under baseline and stress conditions. So, it took a journey of more than 10 years for an interesting phenomenon to evolve to a potentially important epigenomic mechanism.

As early *in vitro* studies had linked the H3S10p-K14ac marks to IEG induction (31), it was thought that this link was universal, i.e., occurring in every *in vitro* and *in vivo* cell system. Our findings in numerous immunohistochemical and ChIP studies do not agree with this notion. It is well-known that induction of the IEGs c-Fos and Egr-1 occurs in a wide range of brain structures after exposure of experimental animals to various acute stressors. These brain structures include the whole neocortex including the prefrontal cortex, hypothalamic, thalamic, and amygdaloid nuclei, hippocampus (DG, CA1), and many pontine and brainstem nuclei (6, 33). The neuroanatomical immuno-localization of H3S10p-K14ac has turned out to be much more restricted with highest levels present in the DG (9, 18, 25, 32). Early studies found only very few H3S10p-K14ac-positive neurons outside the DG such as those in the neocortex, amygdala, and striatum. In more recent experiments, newer generations of antibodies and implementing immuno-staining techniques, which allow better

antibody penetration find stronger staining among neurons in these brain regions (Carter et al., unpublished observations). However, remarkably, ChIP studies have found that only in the DG, H3S10p-K14ac is associated with the *fos* and *egr1* genes (9); thus, in other brain areas this dual histone mark is associated with other, as yet unknown genes. Apparently, in these brain areas histone acetylation and/or histone H3 K4 methylation is sufficient for IEG induction but this still needs to be investigated in detail. As the H3S10p-K14ac histone marks are thought to be associated with hitherto silent genes, several years ago we postulated that the c-Fos and Egr-1 gene promoters in the DG are in a different (condensed?) state than elsewhere in the brain (13, 34). In other words, the IEG promoters in this brain structure require the formation of the H3S10p-K14ac mark to open up (decondense) to allow transcription factor binding and induction of gene transcription (14, 15).

FINDING THE PATH TO THE CHROMATIN

Despite these findings collected over the past 10 years, it remained a mystery how GRs were affecting epigenetic and gene transcriptional changes in dentate neurons in relation to the consolidation of stress-related memories and behavioral responses. It was clear from our studies that GRs in DG neurons play an important role in the phosphorylation of S10 and the acetylation of K14 and possibly other lysine residues at the n-terminal tail of histone H3 molecules within the *fos* and *egr1* gene promoters (9, 18). However, as GRs have no intrinsic kinase and histone acetyltransferase activities, evidently the effects of this steroid receptor annex ligand-dependent transcription factor on H3S10p-K14ac formation were brought about in an indirect manner. Classically, GRs act through glucocorticoid-responsive elements (GREs) within promoter regions of glucocorticoid-responsive genes. Since the transient response in H3S10p-K14ac peaks at 30–60 min after stress, it was considered unlikely that GR-induced genes would be directly involved in modifying histone H3. This thought was strengthened by our observation that an injection of glucocorticoid hormone was ineffective in changing H3S10-K14ac in dentate neurons (25). Moreover, this observation excluded the possibility of a (fast) glucocorticoid effect via membrane-associated GRs. Thus, based on all available data, we postulated the involvement of (an) additional signaling pathway(s).

Since we regard the behavioral immobility response as a learned behavior, we postulated the participation of a pathway typically involved in learning and memory processes, i.e., the NMDA receptor-mediated ERK–MAPK pathway (ERK, extracellular signal-responsive kinase; MAPK, mitogen-activated protein kinase) (35–38). In a series of studies, we indeed found that the NMDA receptor antagonist MK801 and the MEK1/2 (MAPK ERK kinase 1/2) inhibitor SL327 strongly inhibited the forced swimming- and novelty-induced formation of H3S10p-K14ac and IEG expression (32). The effects of the MEK inhibitor indicated the involvement of a MAPK in the signaling pathway. Immunohistochemical analyses indeed showed the transient formation of phosphorylated ERK1/2 (pERK1/2) in DG neurons after forced swimming, but not of phosphorylated p38MAPK, underlining that there is specificity in the recruited MAPK pathway (9). pERK1/2 is however not a histone H3 kinase, thus an intermediary, histone H3 kinase needed to be sought. In collaboration with Dr. Simon Arthur (University of Dundee, UK), we studied mice with a double gene deletion for MSK1/2 (mitogen- and stress-activated kinase 1/2). MSK enzymes can be activated through phosphorylation by pERK1/2 (39). In MSK1/2 knock-out mice, we found virtually an absence of forced swimming-induced H3S10p-K14ac formation and c-Fos induction in dentate granule neurons and a severe impairment of the behavioral immobility response in the re-test (32). Furthermore, Dr. David Sweatt and colleagues at Baylor College (USA) reported in MSK1 gene deleted mice an impaired performance in the Morris water maze and in the contextual fear conditioning paradigm (40). In rats, we found a transient increase in the number of sparsely distributed pMSK1/2 immuno-stained granule neurons in the DG after forced swimming (9). There was no staining of the phosphorylated form of the MSK-related kinases RSK1/2 (pRSK1/2; phosphorylated ribosomal S6 kinase 1/2), which are also substrates of pERK1/2, again underlining specificity. Elk-1 (Ets-like protein kinase) plays an important role in the induction of IEGs like c-Fos and Egr-1 (41–43). *In vitro* work has shown that, upon phosphorylation, for instance by pERK1/2, pElk-1 can bind to the Elk-1 binding site within the SRE(s) [serum response element(s)] and exert trans-activational influences within the *fos* or *egr1* gene promoters (42, 43). pElk-1 has been shown to fulfill these effects through recruitment of the histone acetyl-transferase p300, which after phosphorylation by pElk-1, acetylates nearby histone H3 tails at K14 and other lysine positions (42, 44). After forced swimming, we found an increase in pElk-1 stained DG neurons that time wise paralleled the responses in pERK1/2 and pMSK1/2 (9). Thus, it appears that the phosphorylation and acetylation of histone H3 occur in a coordinated fashion.

Using pharmacological tools and a gene deletion model, we had identified a pathway of interlinked signaling partners that convey extracellular signals (glucocorticoid hormones, glutamate) triggered by environmental challenges (e.g., forced swimming) to the epigenome resulting in gene transcriptional changes in DG neurons. However, the sparse distribution pattern of pERK1/2, pMSK1/2, pElk-1, H3S10p-K14ac, c-Fos, and Egr-1 immunopositive granule neurons within the DG prompted the critical postulate that, in order to build a true signaling cascade, all signaling molecules needed to co-exist in the same neurons. GRs and NMDA receptors are ubiquitous in the DG. Elaborate double immuno-fluorescence analyses provided the final evidence that, within sparsely distributed DG granule neurons, forced swimming and other psychologically stressful challenges indeed activate GRs and the NMDA/ERK1/2/MSK1/2-Elk-1 signaling pathways resulting in H3S10p-K14ac formation and IEG induction (9).

From a functional perspective, it was of critical importance to show that the NMDA/ERK1/2/MSK1/2-Elk-1 signaling pathway is essential for the forced swimming-induced behavioral immobility response. Using the aforementioned pharmacological and gene deletion approaches, we found that any intervention of this signaling cascade resulted in an impairment of the behavioral response (9, 18, 32). Reports of other investigators have provided strong evidence for an involvement of the NMDA receptor, ERK-MAPK signaling, and MSK1 receptors in the consolidation of spatial and emotional memories associated with the Morris water maze

paradigm and contextual fear conditioning (36, 40, 45) (Carter et al., unpublished).

CROSS-TALK OF SIGNALING PATHWAYS: A NOVEL MECHANISM OF GLUCOCORTICOID ACTION

Thus, at this stage, the involvement of a second major signaling pathway, the NMDA/ERK1/2/MSK1/2-Elk-1 pathway, in the forced swimming-induced immobility response, and possibly Morris water maze behavior and contextual fear conditioning, had been resolved. The identification of this second pathway however as yet did not provide insight into the mechanism of action of the GRs on behavior. The outstanding question had remained whether GRs affect behavior through interaction with the ERK MAPK pathway. To address this question, the role of GR activity was investigated in the forced swimming-induced ERK1/2, MSK1/2, and Elk-1 phosphorylation by pre-treating rats with the GR antagonist RU38486 before the stress challenge. The GR antagonist did not affect the stress effect on pERK1/2 but strongly inhibited the formation of pMSK1/2 and pElk-1 in the dentate neurons (9) suggesting that the drug was acting downstream from pERK1/2 and more so that pERK1/2 required activated GRs to phosphorylate MSK1/2 and Elk-1. This is consistent with the earlier notion that these glucocorticoid effects do not involve membrane-associated GRs. Follow-up co-immuno-precipitation studies showed that GR and pERK1/2 indeed undergo physical interactions after forced swimming to facilitate the generation of pMSK1/2 and pElk-1 [**Figure 2**; (9)]. Thus, our data showed evidence for a novel mechanism in which GRs act like a scaffold to facilitate the phosphorylation of MSK1/2 and Elk-1 by pERK1/2 (**Figure 2**). These effects of GRs take place within 15 min after start of forced swimming, thus not as fast as the reported membrane GR-mediated effects (46) but much quicker than the classical genomic effects of this corticosteroid receptor (30–60 min). Thus, we have uncovered a novel non-genomic mode of action of glucocorticoids, which is taking place in the immediate-early time domain after stress (9).

ANXIETY STATUS AS DETERMINANT FACTOR: ROLE OF GABA

The emotional or anxiety status plays a profound role in the impact of psychologically stressful events on physiological, behavioral, and cognitive responses. Accordingly, it is thought that more anxious individuals make stronger memories of stressful events than less anxious individuals. Since many years, great research efforts are vested into the identification of risk factors for the development of PTSD after traumatic experiences. For instance, physical abuse during childhood, thus previous trauma, has been found to be a risk factor for the development of PTSD in Vietnam War veterans (47, 48). More recent work suggests that people who are more anxious have an increased risk for developing PTSD after a traumatic event (49). We were interested to which degree the anxiety status would affect molecular mechanisms in DG neurons known to be involved in memory formation of stressful events. We wanted to explore the role of anxiety by applying three different approaches.

The first approach made use of the rodent's innate fear of open spaces and bright light. We had observed that the mild psychological stressor novelty exposure resulted in an enhanced formation of H3S10p-K14ac and induction of c-Fos in the rat DG (25). The novelty paradigm entailed that the group-housed rat would be individually placed in a cage with new bedding in an unfamiliar room illuminated with a rather bright light source (450–500 lx). For rodents, this is a stressful condition as they instinctively fear predation in well-lit open spaces. We observed that exposing rats to a new cage under light conditions akin those in the holding room (100 lx) led to hardly any rise in H3S10p-K14ac and c-Fos in dentate neurons. However, increasing the light intensity (up to 500 lx) progressively resulted in stronger novelty-induced histone and IEG responses in these neurons (25). Thus, increasing the anxiogenicity of the stressful condition enhanced the epigenetic and gene transcriptional responses in the dentate granule neurons.

Secondly, we addressed the factor anxiety by adopting a pharmacological approach by using the anxiolytic or anxiogenic properties of certain GABAergic drugs. A well-known GABA-related anxiolytic drug is the benzodiazepine Lorazepam. Pre-treatment of rats with an anxiolytic dose of Lorazepam (high doses are sedative) resulted in a complete blockade of the H3S10p-K14ac and c-Fos responses to novelty (50). In contrast, application of the partial inverse agonist FG7142 produced strongly augmented novelty-evoked responses in these histone and IEG marks (50). FG7142 has been shown to increase the excitability of neurons in other parts of the brain to airjet and predator exposure (51, 52) and is known to be strongly anxiogenic in rodents and humans (53–56). The drug lowers GABA-A function and clearly has actions opposite those of Lorazepam. In aggregate, it is clear that GABA plays a major role in the modulating effect of anxiety on responsiveness of dentate neurons to stressful events.

The third approach concerns the application of the voluntary exercise paradigm. Research over the past 15 years has shown that allowing rats and mice access to a running wheel has major beneficial effects for their health and wellbeing. Rats and mice run voluntarily in a wheel during their active period of the day reaching on average a distance of approximately 4 and 6 km per day, respectively (57–59). If this type of voluntary exercise was allowed for several weeks, it proved to have remarkable beneficial effects on a broad range of cellular, physiological, and behavioral processes including neurogenesis in the DG (60–63), the central control of HPA axis responses to psychologically stressful events (57–59, 64), on sleep quality (increased slow wave sleep) (65), and on cognitive, impulsive, and emotional behavior (60, 66). Using the elevated plus-maze, dark–light box, and the novel cage paradigm, long-term exercise was found to have strongly diminishing effects on anxiety levels in both rats and mice (66). Although still somewhat controversial, regular exercise has been shown to have beneficial effects in anxious and depressed patients (67, 68). Therefore, in many countries exercise is presently prescribed to such patients as a co-treatment in addition to the classical pharmacological and behavioral therapies. We investigated whether exercise would impact on stress-induced behavior. We found that exercised rats showed remarkably different behavior in the novel cage paradigm than sedentary animals (69). When sedentary rats are placed alone in a new cage (lights: 500 lx), they explore the novel environment for the full 30 min the test lasts. However, exercised rats explored the new cage for only 10–15 min after which they laid down to rest or sleep, i.e., the normal behavior a rat displays during the daytime (69). Thus, apparently exercised animals

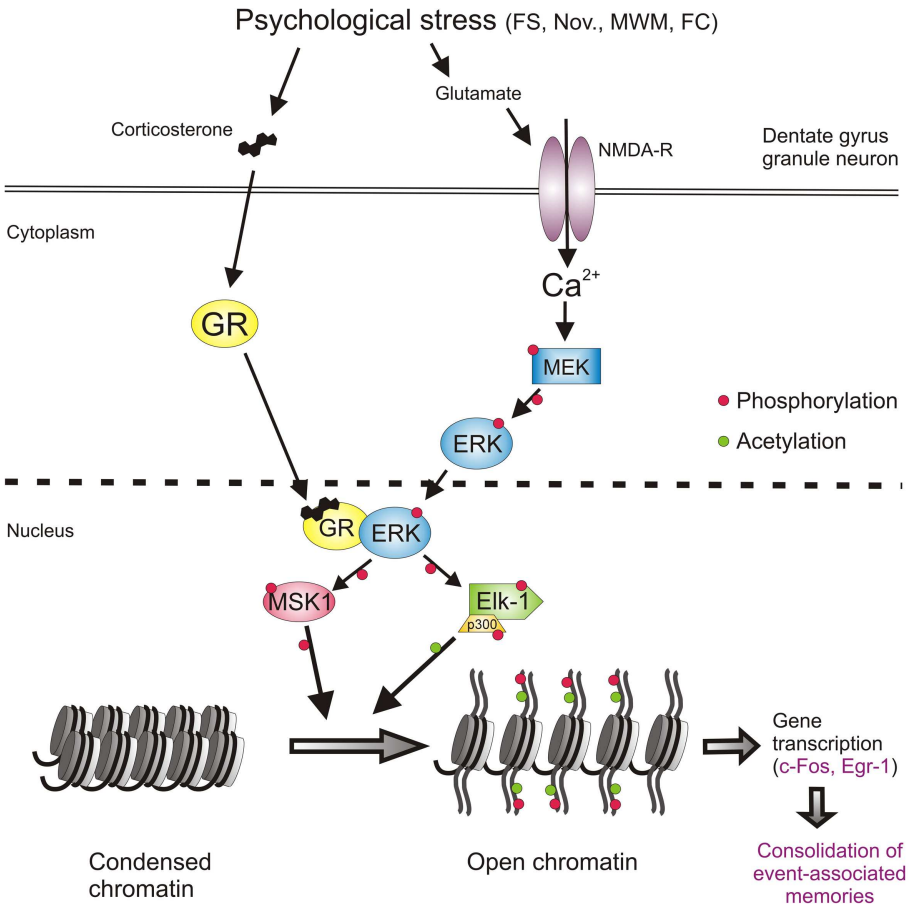

FIGURE 2 | Psychological stress-activated signaling pathways in dentate gyrus granule neurons driving epigenetic modifications underlying induction of gene transcription and the consolidation of behavioral responses and memory formation. Psychological stress evokes the concomitant activation of the GR and NMDAR-ERK-MAPK pathways. The concomitant activation of ERK1/2 and GR and their subsequent physical interaction facilitates the ability of pERK1/2 to phosphorylate MSK1/2 and Elk-1. Activation of these nuclear kinases results in the phosphorylation and acetylation of histone H3 (H3S10p-K14ac), which drives chromatin remodeling thereby allowing the gene transcription of IEGs like *c-fos, egr1*, and many other genes. The induction of gene transcription is critical for the consolidation of memory formation associated with the endured event. See text for references of studies supporting this concept. FS, forced swimming; Nov., novelty exposure; MWM, Morris water maze training; FC, contextual fear conditioning.

much quicker reach the conclusion that the new environment is safe, which corresponds with their lower anxiety level and possibly enhanced cognition. The reduced anxiety levels in the exercised rats may be due to changes in their GABAergic system. We reported that in addition to distinct changes in the expression of GABA-A receptor subunits (e.g., the extra-synaptic receptor associated delta and alpha-5 subunits), long-term exercise resulted in an increased gene transcription of the GABA synthesizing enzyme GAD67 (70). Furthermore, our recent preliminary findings suggest that GABA synthesis capacity is increased in the DG of exercised rats (Kersanté et al., unpublished observations).

We investigated changes in ERK-MAPK signaling and c-Fos expression in the DG after long-term voluntary exercise. We found that 4 weeks of wheel running resulted in a significant attenuation of the forced swimming-induced increases in the pERK1/2, pMSK1/2, and c-Fos (Collins et al., unpublished observations). Thus, exercised rats show reduced ERK-MAPK and IEG responses to forced swim stress, which may be a consequence of the enhanced GABAergic inhibitory tone in the DG of these animals (50).

Together, this work strongly supports the notion that the anxiety state and the state of the GABAergic system play a pivotal role in the responsiveness of DG granule neurons to psychological stress. In molecular terms, this responsiveness is translated into the likelihood of initiation of ERK-MAPK signaling, epigenetic changes, and induction of (IEG) gene transcription. The findings also suggest that anxiety acts upon these molecular mechanisms through modulation of the GABAergic tone arising from local interneurons in the DG. The GABAergic tone is regulated locally by adjustments in GABAergic synthesis and release capacity, GABA-A receptor subunit composition as well as through afferent inputs from other regions of the brain (see below).

The observation that particularly fearsome and emotional events impact strongly on the extent of activation of dentate

granule neurons may underlie the well-known phenomenon that such events are typically very strongly stored into memory, often life-long. In terms of behavioral adaptation and from an evolutionary perspective this makes great sense. Keeping track of potential predators, conspecific enemies, dangerous places, and other threats is crucial to stay safe and avoid violence and predation. Evidently, the level of anxiety awareness and GABAergic control need to be tightly regulated to remain healthy and safe. Hyper-anxiety/low GABAergic control may be profoundly debilitating (humans: social isolation, incapability; animals: social isolation, starvation) whereas low anxiety/high GABAergic control may be dangerous to the individual (humans: carelessness; accident-prone, sensation-seeking behavior: injuries, death; animals: predation). In addition to these state-dependent variables affecting the health condition of humans and animals, repeated challenges leading to chronic stress as well as gravely traumatic life events (e.g., rape, abuse, extreme violence and horror, like in war situations, and other near-death experiences) can lead to depressive and anxiety disorders like PTSD (71, 72) possibly through damage and/or dysfunction of the DG and other parts of the hippocampal formation.

ROLE OF AFFERENT INPUT TO THE DENTATE GYRUS

In addition to local (GABAergic) mechanisms regulating DG excitability also extra-hippocampal, afferent input to this hippocampal region is of principal importance. It has been established that information flow through the hippocampus is modulated by various afferent inputs from subcortical regions of the brain including the septum (73), locus coeruleus (74), raphe nuclei (75, 76), amygdala (77, 78), and the supra-mammillary area (SMA) in the hypothalamus (79–82).

THE SUPRA-MAMMILLARY AREA, A HYPOTHALAMIC REGION INVOLVED IN INTEGRATING COGNITIVE AND EMOTIONAL BEHAVIOR

The hippocampal formation receives substantial afferent projections from the SMA, which are channeled through the fimbria–fornix. This input has a strong influence on hippocampal theta rhythms and is therefore thought to play an important role in hippocampus-dependent cognitive functions and emotional behavior. Lesions of the mammillary area, including the SMA, have been reported to result in impaired spatial learning and memory in several behavioral tasks including the water maze (83–86). The neuroanatomy of the SMA–hippocampus connection is complex and has been investigated for many years. Although the neuroanatomy has not been fully clarified, the projections seem to consist of glutamatergic and GABAergic fibers, which predominantly innervate the DG and to a lesser extent the CA2/CA3a region of the hippocampus (87–89). Physiologically, the main effect of SMA stimulation is the facilitation of perforant path-elicited population spikes in the DG (80, 90–92). This facilitation has been thought to result from a disinhibition mechanism due to GABAergic SMA–DG afferents inhibiting local dentate GABAergic interneurons (80). Such afferents on DG interneurons have however not been found and it seems that virtually all SMA-DG afferent fibers project to granule neurons in the DG (88). Presently, supported by anatomical studies (88, 93–95), it is thought that the SMA potentiates population spikes evoked by perforant path stimulation in the DG via direct excitatory glutamatergic synaptic neurotransmission upon granule neurons (95). Yet, Nakanishi et al. reported that SMA-evoked facilitation of perforant path stimulated EPSP spikes in DG was blocked by the GABA-A blocker picrotoxin leaving the possibility open for a disinhibitory role of GABAergic interneurons in granule neuron excitability (92). It seems that, using both excitatory and inhibitory afferent inputs, the SMA plays a pivotal role in facilitating information flow in the DG in a behavior-dependent manner (96).

INTERPLAY OF THE SMA AND THE AMYGDALA

Evidence has been accumulating that a role of the SMA in controlling information flow in the hippocampus also affects the influence of the amygdala on hippocampus function. Since many years, it has been known that the amygdala plays a pivotal role in hippocampus-mediated learning and memory processes associated with emotion. Although neuroanatomically a link between the amygdala and DG has not been clarified yet, physiological research has provided ample evidence for the existence of such link. It has been shown that lesioning or functionally inhibiting the basolateral amygdala attenuates long-term potentiation (LTP) in the DG (97, 98). Stimulation of this amygdala region facilitates perforant path-DG synaptic responses (78). Furthermore, high-frequency stimulation of the medial amygdala evokes a long-lasting potentiation of perforant path-DG population spikes (99). McGaugh et al. reported functional neuroanatomical evidence that injection of NMDA into the amygdala evokes the induction of c-Fos in the DG (100, 101).

Thus, afferent projections from the SMA and the amygdala to the DG play an important facilitatory role in merging the influence of anxiety/emotionality with the multimodular sensory information flow through the DG. This physiological mechanism appears to be instrumental in facilitating the formation of memories of emotionally charged life events. At the DG cellular level, the coordinated inputs from the SMA and amygdala resulting in an enhanced excitability of dentate neurons may translate into an enhanced likelihood of NMDAR-mediated excitation of dentate granule neurons resulting downstream into activation of signaling, epigenetic, and gene transcriptional changes known to be required for the consolidation of memory formation. Clearly, research is required to provide substance to this notion.

SIGNIFICANCE OF H3S10p-K14ac FORMATION FOR INDUCTION OF GENE TRANSCRIPTION

The induction of the IEGs, c-Fos and Egr-1, in DG granule neurons after psychological stressors such as forced swimming and novelty requires the formation of dual histone mark H3S10p-K14ac (9, 25, 32). However, although *in vitro* research suggests that the association of this dual histone mark with IEG gene induction is a general phenomenon, in the brain *in vivo* this is not the case. Using ChIP, we found that the dual histone mark is only associated with the c-Fos and Egr-1 promoter region in the DG but not in the neocortex (9). In view of evidence that the H3S10p-K14ac histone mark is associated with the opening of dormant genes (28, 30, 31), we have concluded that under baseline conditions the c-Fos and Egr-1 genes and possibly many other genes in the DG are in a different,

i.e., condensed, state and require the formation of the dual histone mark in their gene promoters for de-condensation and gene transcription (13–15, 34, 102).

Presently, it is unknown why IEG induction in DG neurons, as opposed to other neurons in the brain, is critically dependent of formation of H3S10p-K14ac. Possibly, as part of the physiological, sparse activation scheme applied in the DG, gene induction in dentate neurons is required to be strictly regulated, apparently to safeguard that after an environmental challenge only a few percent of DG neurons are responding. Thus, dentate neuron activation and function is controlled at several levels including the tonic GABAergic control and other afferent (SMA, amygdala) control at the cellular level, the NMDA receptor-mediated Ca^{2+}/ERK-MAPK requirement at the signaling level and the requirement of H3-S10pK14ac-driven chromatin remodeling at the molecular level. In addition to the dual histone mark, our recent work suggests that also the DNA methylation status of distinct CpGs within the *fos* and *egr1* gene promoters play a critical role in the transcriptional activity at these genes after stress (Saunderson et al., in preparation). This additional epigenetic mechanism adds another level of molecular control of IEG expression in DG neurons.

Recently, we postulated that these histone modifications and the opening of the chromatin structure are needed to provide transcription factors like CREB access to their DNA binding sites (14, 15). CREB is a well-known trans-activator of c-Fos and Egr-1 gene transcription. Our concept attempts to explain the observation that, although CREB phosphorylation occurs ubiquitously in the DG after stressful challenges like forced swimming (6), c-Fos and Egr-1 are only expressed in those DG granule neurons in which H3S10p-K14ac has been generated (9, 25, 32).

To obtain deeper insight into the functional implications of the H3S10p-K14ac mark in dentate neurons, we have started ChIP studies in combination with Illumina next-generation sequencing (ChIP-Seq). This genome-wide screen will deliver detailed knowledge about the identity of all genes specifically associated with the dual histone mark, other histone marks as well as those genes, which show binding of distinct transcription factors within their gene promoters. Thus, in other words, this state-of-the-art approach will inform us about which genes in the activated dentate neurons are specifically involved in adaptive changes in these neurons after a stressful challenge. This information will help to obtain insight into the functional changes occurring in these neurons and furthermore will assist in the elucidation of the functional properties of as yet unknown genes.

OUTLOOK

As mentioned at the beginning of this text, adaptation to stressful events in our lives, which includes the formation of memories of such events, is of critical importance to maintain health and well-being. Anxiety disorders like PTSD and major depressive disorder are thought to be the consequence of disruptions and impairments in this adaptive process. The likelihood of developing this kind of mental disorders is higher in individuals who have been subject to early life abuse or neglect, or have endured massive, acute traumatic events or chronic psychological stress. The observation that "only" 10–20% of people develop a mental disorder under such conditions suggests the involvement of genetic factors as well as "phenotypic" factors (e.g., age, lifestyle, socio-economic status), which determine an individuals' resilience to stress.

The DG plays a pivotal role in the encoding of incoming sensory and other information from the entorhinal cortex involving pattern separation (103, 104) that enables the CA3 to utilize this information for further processing and integration, which is key to the formation of event-associated memories. This NMDAR-dependent process in sparse dentate granule neurons evokes long-term molecular and cellular changes in these neurons, which subserve long-lasting changes in functional properties of these cells. Our work has helped to gain insight into the signaling, epigenetic, and gene transcriptional mechanisms evoked in these dentate neurons after a psychologically stressful challenge. Previously, we have suggested that these mechanisms act like a molecular switch allowing hippocampal information processing and thereby the consolidation of memories associated with the challenge (13). The generation of the dual histone mark H3S10p-K14ac, driven by concomitant activation of the GR and NMDAR/ERK1/2/MSK1-Elk-1 pathways, plays a central role in kick-starting gene transcription required for long-term changes in neuron function. This concept provides the framework for the identification and investigation of the gene products involved beyond the IEGs c-Fos and Egr-1. Furthermore, it invites investigating the role of afferent input from subcortical brain structures in the regulation of DG granule neuron function at the molecular level. Together, this research opens up the opportunity for preclinical and clinical studies on the pathophysiological significance of the participating genes and thereby hopefully for drug development to combat stress-related mental disorders.

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Therapeutic action of fluoxetine is associated with a reduction in prefrontal cortical miR-1971 expression levels in a mouse model of posttraumatic stress disorder

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MicroRNAs (miRNA) are a class of small non-coding RNAs that have recently emerged as epigenetic modulators of gene expression in psychiatric diseases like schizophrenia and major depression. So far, miRNAs have neither been studied in patients suffering from posttraumatic stress disorder (PTSD) nor in PTSD animal models. Here, we present the first study exploring the connection between miRNAs and PTSD. Employing our previously established PTSD mouse model, we assessed miRNA profiles in prefrontal cortices (PFCs) dissected from either fluoxetine or control-treated wildtype C57BL/6N mice 74 days after their subjection to either a single traumatic electric footshock or mock-treatment. Fluoxetine is an antidepressant known to be effective both in PTSD patients and in mice suffering from a PTSD-like syndrome. Screening for differences in the relative expression levels of all potential miRNA target sequences of miRBase 18.0 by pairwise comparison of the PFC miRNA profiles of the four mouse groups mentioned resulted in identification of five miRNA candidate molecules. Validation of these miRNA candidates by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) revealed that the therapeutic action of fluoxetine in shocked mice is associated with a significant reduction in mmu-miR-1971 expression. Furthermore, our findings suggest that traumatic stress and fluoxetine interact to cause distinct alterations in the mouse PFC miRNA signature in the long-term.

Keywords: miRNA, miR-33, miR-1971, PTSD, PTSD mouse model, prefrontal cortex, SSRI

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a debilitating anxiety disease occurring in about 2–9% of individuals after their exposure to life-threatening events like severe accidents, sexual abuse, combat, or natural catastrophes (1, 2). Although selective serotonin reuptake inhibitor (SSRIs) antidepressants like fluoxetine are currently the first line choice in PTSD drug treatment (3, 4), the response rates to SSRI treatment rarely exceed 60% and less than 20–30% of SSRI-treated PTSD patients achieve full remission (5). This unsatisfactory situation together with the fact that there is currently no drug available that specifically tackles PTSD core symptoms (3, 5), namely re-experiencing of traumatic memories, nervous hyperarousal, and avoidance of trauma-related cues (6) stresses the urgent need for development of novel PTSD-specific drugs and hence for elucidation of the, as yet insufficiently explored, molecular basis of PTSD. Epigenetic mechanisms increasingly emerge to play a role in PTSD pathobiology (7), i.e., it was recently discovered that allele-specific DNA demethylation of *FKBP5*, a potential PTSD biomarker (8), mediates gene × childhood trauma interactions (9). Furthermore, epigenetic regulation of immune-system associated molecules (10) and of catechol-*O*-methyltransferase (COMT), an enzyme which is critical for regulation of synaptic dopamine, was reported to be altered in PTSD patients (11). Besides DNA methylation and histone modifications, some authors consider small non-coding RNAs, like the about 22 nucleotides (nt) long miRNAs, as epigenetic regulators (12, 13). However, the view of miRNAs as regulators of epigenetic processes as well as reports on the epigenetic regulation of miRNA expression are more common (14, 15). miRNAs are well conserved in eukaryotic organisms (16) and play a pivotal role in regulation of posttranscriptional gene expression (12). They are encoded by eukaryotic DNA and function via base-pairing with complementary sequences of mRNA molecules through rapid mRNA decay and direct translational repression (17). MiRNAs have been associated not only with cancer (14) and autoimmune diseases (18) but also with psychiatric disorders like schizophrenia, autism (19), major depression (20), and anxiety diseases like panic disorder and specific phobias (21). In mice, expression of miR-128b was found to be increased in infralimbic prefrontal cortices (PFCs) in response to fear extinction training (22) which is considered to model exposure-based therapy (23), a psychotherapeutic strategy applied *inter alia* in PTSD patients (24). Furthermore, there is much evidence for miRNAs to play an important role in relation to the epigenetic tuning of the stress response (25, 26). For example, stress was shown to up-regulate mi34c expression in mouse amygdala and, moreover, lentivirally

overexpressed mi34c was reported to induce anxiolytic-like behavior after challenge (27). Interestingly, to the best of our knowledge, miRNA regulation, expression, and function have so far not been studied at all in PTSD, neither in PTSD patients nor in PTSD animal models. Here, we present the first study exploring the connection between miRNAs and the PTSD-like syndrome in rodents. Using a miRNA microarray, we analyzed miRNA profiles in our previously established mouse model for PTSD (28, 42). In detail, we compared miRBase 18.0 based miRNA profiles in PFC samples of four groups of mice, i.e., footshocked and non-footshocked mice which were either fluoxetine-treated or untreated. We chose the PFC for miRNA profile analysis since this brain region was found to be reduced in volume (30) as well as altered in function (31, 32) in PTSD patients. Moreover, since in the PTSD model studied here we found shocked mice to exhibit an increased conditioned fear response, the notion that the PFC, beyond its known function in fear extinction (33), increasingly emerges to play a role in fear conditioning (33, 34) further sparked our interest in this brain region. In addition, prefrontal cortical miRNA expression levels have been reported to be altered in other psychiatric disorders: for instance, let-7d was shown to be up-regulated in the PFC of spontaneous hyperactive rats, an animal model for attention deficit hyperactivity disorder (ADHD) (35), and miR-195 was demonstrated to fine-tune regional levels of brain derived neurotrophic factor (BDNF) in the PFC of schizophrenic patients (36).

MATERIALS AND METHODS

ANIMALS

All experimental procedures were approved by the Committee on Animal Health and Care of Upper Bavaria (Regierung von Oberbayern), Germany (approval ID-AZ: 55.2-1-54-2531-41-09) and were conducted according to the current regulations for animal experimentation in Germany and the European Union (European Communities Council Directive 86/609/EEC). Twenty-three days old male C57BL/6NCrl mice purchased from Charles River GmbH (Sulzfeld, Germany) were housed in groups in the animal facility of the Max Planck Institute (MPI-P) for 6 weeks under an inverse 12:12 h light-dark cycle (lights off: 09:00 a.m.) with food and water *ad libitum*.

PTSD MOUSE MODEL

Experiments were performed during the activity phase of the mice, i.e., between 9:30 a.m. and 6:00 p.m., employing our established PTSD mouse model which we described in detail previously (28, 29). Briefly, 10-week-old male C57BL/6NCrl mice were subjected to a single 1.5 mA electric footshock for 2 s or mock treatment (exposure to shock chamber, the latter is termed "shock context" or "shock chamber" in the following). Beginning the day after footshock or mock treatment, half of the footshocked and half of the mock-treated mice received oral fluoxetine treatment ($n = 6$ per group). Thus, we compared four groups of mice, i.e., footshocked and mock-treated mice, which were either fluoxetine (Ratiopharm, Ulm, Germany) or vehicle-treated; these groups are termed "no-shock-vehicle," "no-shock-fluoxetine," "shock-vehicle," and "shock-fluoxetine" in the following. Fluoxetine was administered in drinking water in a dose of 20 mg/kg/day for 28 days. The control group received drinking water only. On day 28 after footshock or mock treatment, fluoxetine efficacy was assessed by evaluation of their generalized fear response for 60 s during the presentation of a neutral tone (80 dB, 9 kHz) in a neutral context. Subsequently, the dose of fluoxetine was halved (10 mg/kg/day) and treatment was further continued for 3 days until discontinuation on day 31. Then, 59–60 days after footshock or mock treatment, hyperarousal was assessed by evaluation of their acoustic startle response. In addition, their generalized fear response was analyzed by monitoring their freezing behavior upon subsequent exposure to a neutral experimental context and to an experimental context similar to the shock chamber. Finally, the conditioned fear response of the mice was assessed by measuring their freezing behavior during (re-)exposure to the shock chamber. Video-taped animal behavior was rated off-line by a trained observer who was blind to the experimental conditions. Statistical analysis of behavioral data was performed using two-way ANOVA and Bonferroni *post hoc* tests.

RNA EXTRACTION

Seventy four days after footshock or mock treatment, mice were sacrificed by cervical dislocation and PFCs were dissected ($n = 6$ per group). Total RNA was extracted employing the TRIzol® protocol following the manufacturer's instructions (Invitrogen, Paisley, UK). Extracted total RNA was resolved in nuclease free water. Concentrations of total RNA were assessed spectrophotometrically with a Nanophotometer (Implen GmbH, Munich, Germany). RNA integrity was assured by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) both in our laboratory and in the laboratory of the microarray service provider (Exiqon A/S, Vedbaek, Denmark). RNA integrity numbers were $\geq$8.90 throughout the samples and all samples exhibited clear 18S and 28S RNA peaks in Bioanalyzer profiles.

miRCURY LNA™ miRNA MICROARRAY PROFILING

RNA samples ($6 \times 4 = 24$ samples) were shipped from the MPI-P in Munich to the microarray service provider Exiqon (Exiqon A/S, Vedbaek, Denmark) where all miRNA microarray experiments were performed. Accordingly, the chapter at hand (description of miRNA microarray procedure) is based on information provided by Exiqon (Exiqon A/S, Vedbaek, Denmark): 600 ng of total RNA extracted from samples were labeled with fluorescent Hy3™ and 600 ng of total RNA from reference probe with fluorescent Hy5™ using the miRCURY LNA™ miRNA Hi-Power Labeling Kit (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's protocol. The Hy3™ -labeled samples were mixed pairwise with a Hy5™ -labeled reference probe and hybridized to the miRCURY LNA™ miRNA Array 7th Gen (Exiqon A/S, Vedbaek, Denmark) which contains capture probes targeting all miRNAs registered in the miRBase 18.0 (human, mouse, or rat)[1] as well as viral miRNAs related to these species. Hybridization was performed according to the miRCURY LNA™ miRNA Array Instruction manual (Exiqon A/S, Vedbaek, Denmark) using a Tecan HS4800™ hybridization station (Tecan Austria GmbH, Salzburg, Austria).

[1]www.mirbase.org

After hybridization, microarray slides were scanned and stored in an ozone free environment (ozone below 2.0 ppb) in order to prevent potential bleaching of fluorescent labels. The miRCURY LNA™ miRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA). Image analysis was carried out with ImaGene® 9 miRCURY LNA™ miRNA Array Analysis Software (Exiqon A/S, Vedbaek, Denmark).

MICROARRAY DATA PROCESSING

Pre-processed microarray data was provided by Exiqon (Exiqon A/S, Vedbaek, Denmark). Accordingly, the description of microarray data processing is based on information provided by Exiqon: Signal intensity was the basis of data filtering. Background correction of quantified signals was performed via subtraction of the median global background from the median local background from the intensity of signals (Normexp with offset value 10) and resulted in the exclusion of two samples of the experimental group "no-shock-fluoxetine" (**Figures 3B** and **4**). Normalization of data was performed with the global Lowess (locally weighted scatterplot smoothing) regression algorithm (37). All calculations have been performed using the software R/bioconductor employing mainly the limma package. Comparisons of miRNA expression values between experimental groups were performed using moderated *t*-statistics with standard errors moderated across genes, i.e., shrunk toward a common value, using a simple Bayesian model. This has the effect of borrowing information from the ensemble of genes to aid with inference about each individual gene (38). *P*-values were corrected for multiple testing by the Benjamini and Hochberg adjustment method to control for false positive results.

With the corrected *p*-values delivered by Exiqon (Exiqon A/S, Vedbaek, Denmark), we performed unsupervised hierarchical clustering analyses (HCA) in which we included the top 50 miRNA candidates with the lowest corrected *p*-values. HCA results are depicted in heatmaps which we generated by a web-based tool provided by the Los Alamos National Laboratory HIV sequence database[2]. For HCA, the complete-linkage method together with the Euclidean distance measure was employed. Complete-linkage clustering (by Euclidean distance) between sample subsets is represented by dendrograms (**Figures 2–4**).

[2]http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html

REVERSE TRANSCRIPTASE QUANTITATIVE PCR

For reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), which was performed at the MPI-P, we employed either pre-designed LNA™ PCR primer sets for miRCURY LNA™ Universal RT microRNA PCR or Custom LNA™ PCR primers (UniRT) (Exiqon A/S, Vedbaek, Denmark). A list of all primer sets and their respective target sequences used is provided in **Table 1**. We used the miRCURY LNA™ miRNA PCR system first strand synthesis kit for poly-adenylation (poly-A-tailing) and reverse transcription (input of total RNA: 100 ng) according to the manufacturer's protocol (Exiqon A/S, Vedbaek, Denmark). Then, 1 μl of 1:80 diluted cDNA was amplified by RT-qPCR in 5 μl SYBR Green PCR master mix containing 0.25 mM of LNA™ miRNA specific primer sets (**Table 1**). The total reaction volume was 10 μl. RT-qPCR was performed on the LightCycler® 480 instrument (Roche Diagnostics, Penzberg, Germany). Each sample was analyzed in duplicate in every run, i.e., for each miRNA candidate tested. Cycling conditions were as follows: denaturation step 95°C 5 min followed by 45 loops of a two-segment amplification step (95°C, 30 s, 62°C, 1 min). A standard curve was generated for each individual plate assay with 1:10, 1:100, and 1:1000 dilutions and PCR efficiencies were calculated. C_p values were obtained with the software provided by the manufacturer (Roche Diagnostics, Penzberg, Germany). MiRNA entities for normalization were selected via NormFinder analysis based on microarray data (39) and mmu-miR-100-5p was used for normalization. Relative expression was calculated by the $\Delta\Delta C_t$ method (40).

miRNA TARGET PREDICTION AND GENE ONTOLOGY ANALYSIS

Materials and methods for miRNA target prediction and gene ontology (GO) analysis are described in detail in the Section "Results."

RESULTS

FLUOXETINE COUNTERACTS THE LONG-LASTING PTSD-LIKE SYNDROME IN MICE

To analyze the impact of traumatic stress on miRNA profiles in mouse PFC, we employed a well-established mouse model for PTSD that we (28, 29, 41–43) and also other research groups used, at least in slightly modified ways (44–46), for previous experiments. The electric footshock-elicited murine PTSD-like syndrome can be effectively counteracted by the orally administered SSRI antidepressant fluoxetine (28, 29) and, as we published

Table 1 | List of primer sets used for RT-qPCR.

Target name	Product no./design ID (custom)	Target miRNA sequence
mmu-miR-33-5p	204632	GUGCAUUGUAGUUGCAUUGCA
mmu-miR-100-5p	204133	AACCCGUAGAUCCGAACUUGUG
mmu-miR-1971	206999 (custom)/design ID 212160	GUAAAGGCUGGGCUGAGA
mmu-miR-1947-3p	206999 (custom)/design ID 212154	GCACUGAGCUAGCUCUCCCUCC
rno-miR-3559-3p	206999 (custom)/design ID 212147	AUGUAGUACUGAGUCUGUCGUG
ebv-miR-BART8-3p	206999 (custom)/design ID 212150	GUCACAAUCUAUGGGGUCGUAGA

We employed either pre-designed LNA™ PCR primer sets for miRCURY LNA™ Universal RT microRNA PCR or Custom LNA™ PCR primers (UniRT) (Exiqon A/S, Vedbaek, Denmark). The primer sets are designed for detection of the respective target sequences.

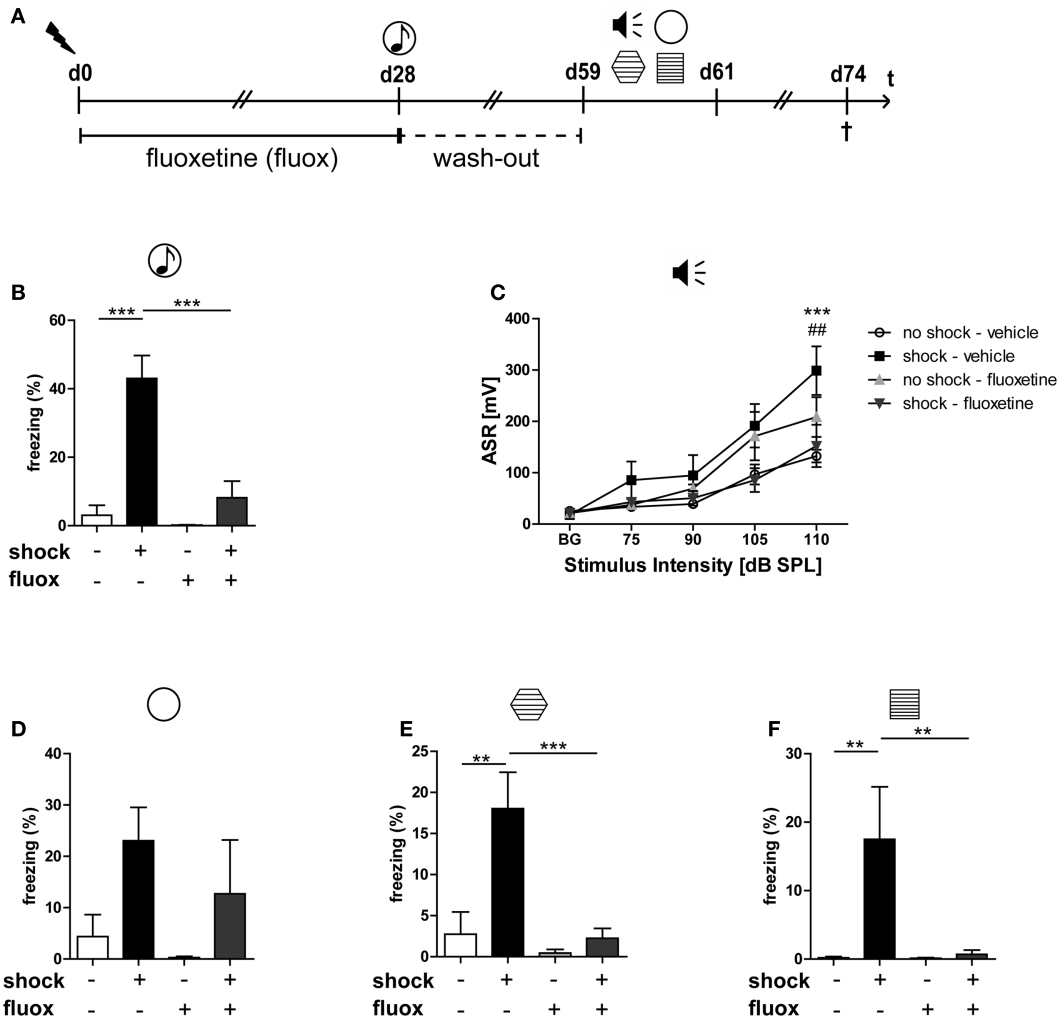

FIGURE 1 | Fluoxetine counteracts the long-lasting PTSD-like syndrome in mice. Male C57BL/6NCrl mice ($n = 6$ per group) were either subjected to a single electric footshock ("shock") or, mock treatment ("no-shock"). Subsequently, both shocked and non-shocked mice were treated with either fluoxetine (20 mg/kg/day) ("fluoxetine") or, for control, with drinking water ("vehicle") for 28 days **(A)**. On day 28 after footshock or mock-treatment their freezing response to a neutral tone was assessed in a neutral experimental context (generalized fear response) **(B)**. On day 29, the dose of fluoxetine was halved (i.e., 10 mg/kg/day) prior to treatment discontinuation on day 31. On days 59–61, PTSD-like behavior was analyzed: first, we assessed the intensity of the acoustic startle reflex (ASR) in response to white noise pulses of 50 dB (background, BG) and 75, 90, 105, and 115 dB **(C)**. Then, we evaluated the generalized fear response by assessment of the freezing response both in a neutral experimental context **(D)** and in a grid context similar to the shock chamber **(E)**. Finally, the conditioned fear response was analyzed by evaluation of the freezing response in the shock context (re-exposure to shock chamber) **(F)**. Freezing duration was assessed for a total of 3 min. The absolute time of immobility except respiratory movements was normalized to this 3 min observation interval (Freezing [%]). Presented data are means ± SEM. Statistical analysis was performed using two-way ANOVA and Bonferroni *post hoc* tests. Statistical significance of Bonferroni *post hoc* tests is indicated, for comparison of the groups "no-shock-vehicle" versus "shock-vehicle" by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; respectively; comparison of groups "shock-vehicle" versus "shock-fluoxetine" by $\#\#p < 0.01$. See Section "Results" for statistical data.

recently, lasts at least until day 60 after shock application (28). First, we had to re-establish the behavioral syndrome-inducing effect of footshock and the relieving action of fluoxetine in the mouse cohort studied here: in contrast to our expectations (28, 29), and despite a significant effect of shock ($F_{1,20}$ shock = 5.696, $p = 0.027$), the relative increase of the generalized fear response of shocked mice in the *neutral* context was not statistically significant after Bonferroni correction on day 60 (**Figure 1D**) but at least on day 28 (**Figure 1B**, $t = 6.461$, $p < 0.001$). The results of the other behavioral experiments turned out as expected: on day 59/60 after their subjection to shock, in comparison to mock-treated control mice, shocked mice exhibited a significantly stronger generalized fear response (in a context similar to the shock chamber) (**Figure 1E**, $t = 4.058$, $p < 0.01$) as well as more pronounced acoustic startle (**Figure 1C**, $t = 4.468$, $p < 0.001$) and conditioned fear responses (**Figure 1F**, $t = 3.609$, $p < 0.01$). Moreover, fluoxetine treatment drastically reduced trauma-mediated behavioral changes (**Figure 1B**: $t = 5.630$, $p < 0.001$, **Figure 1C**: $t = 3.939$,

$p < 0.01$, **Figure 1E**: $t = 4.193$, $p < 0.001$, **Figure 1F**: $t = 3.505$, $p < 0.01$).

The behavioral consequences of stress exposure make this mouse model an animal model of PTSD, not the type or intensity of the stressor applied. The relatively increased conditioned and generalized fear responses in footshocked mice mirror the PTSD-associated avoidance behavior in humans: In most PTSD patients, the aversive avoidance of trauma-related reminders generalizes over time in sense that someday also trauma-unrelated cues suffice to elicit an intense avoidance anxiety. Moreover, the relatively increased startle response in footshocked mice has been repeatedly described also in PTSD patients (47–49). Hence, it reflects trauma-elicited nervous hyperexcitability in both men and mice. Other PTSD animal models employ more intense stressors in order to better model their traumatizing nature (50, 51).

TRAUMATIC FOOTSHOCK *PER SE* DOES NOT SIGNIFICANTLY ALTER MOUSE PFC miRNA PROFILES IN THE LONG-TERM

To avoid molecular analyses to be influenced by acute effects of the behavioral testing procedure, we harvested mouse brains 2 weeks after behavioral analyses. For preparation of total RNA and subsequent miRNA profile analyses, PFCs were dissected from six mice per group. With the aim to identify miRNA candidates regulated by traumatic stress and/or by fluoxetine treatment, we subjected all of these 24 PFC total RNA samples to miRNA microarray analysis. After background correction and normalization, expression values were subjected to pairwise *t*-testing (no-shock-vehicle versus shock-vehicle; shock-vehicle versus shock-fluoxetine; no-shock-fluoxetine versus shock-fluoxetine; no-shock-vehicle versus no-shock-fluoxetine) and the resulting *p*-values were Benjamini–Hochberg corrected. Then, miRNAs were ranked by corrected *p*-values and the resulting top 50 candidates, i.e., the miRNAs with the lowest *p*-values, were subjected to unsupervised HCA. We performed four HCAs in total (**Figures 2–4**).

First, we looked for miRNAs regulated by traumatic footshock: unsupervised HCA of footshocked versus non-shocked groups (both vehicle-treated) revealed that samples clustered, with one exception, according to treatment by their miRNA expression values (**Figure 2**). However, pairwise comparison of miRNA expression profiles showed that no miRNA was significantly differentially expressed between these two groups. Thus, traumatic footshock causes a long-lasting PTSD-like syndrome in mice (**Figure 1**) but does not significantly alter long-term miRNA expression in mouse PFC (**Figure 2**).

FLUOXETINE TREATMENT SIGNIFICANTLY ALTERS THE EXPRESSION OF SEVERAL miRNAs IN THE PFC OF SHOCKED MICE

Then, we looked for the influence of fluoxetine treatment on miRNA profiles of shocked mice: unsupervised HCA of shocked fluoxetine-treated (shock-fluoxetine) versus shocked vehicle-treated (shock-vehicle) groups revealed that samples clustered perfectly according to treatment (**Figure 3A**). Moreover, comparison of these two groups, i.e., the shock-vehicle versus the shock-fluoxetine group, revealed, that therapeutic (**Figure 1**) fluoxetine treatment significantly reduced the relative expression of two miRNA candidates analyzed, namely of rno-miR-3559-3p [fold change (FC) 0.29, corrected *p* (corr. *p*) < 0.003] and of mmu-miR-1971 (FC 0.82, corr. $p < 0.05$) (**Figure 3A**, highlighted in bright pink) and furthermore decreased the expression of two other miRNAs [at least on the level of a trend toward statistical significance (i.e., with $p < 0.1$)], namely the expression levels of ebv-miR-BART8-3p (FC 0.53, corr. $p < 0.06$) and of mmu-miR-1947-3p (FC 0.67, corr. $p < 0.06$) (**Figure 3A**, highlighted in bright blue).

Next, to further dissect the individual contributions of traumatic stress and fluoxetine treatment on mouse PFC miRNA signatures, we compared the shock-fluoxetine group to the no-shock-fluoxetine group (**Figure 3B**) and to the no-shock-vehicle group (**Figure 4**). Two samples of the no-shock-fluoxetine group had to be excluded from microarray analysis during array data processing. HCA of the no-shock-fluoxetine group versus the shock-fluoxetine group showed that the two groups did not cluster correctly according to treatment. However, pairwise comparison of these two groups revealed that, the relative expression of mmu-miR-33-5p was enhanced, at least with a statistical trend (FC 1.26, corr. $p < 0.07$) and the relative expression of rno-miR-3559-3p was decreased, also with a statistical trend (FC 0.80, corr. $p < 0.07$) in PFC of shock-fluoxetine mice (**Figure 3B**, both highlighted in bright blue). Although the HCA of the no-shock-fluoxetine group versus the no-shock-vehicle group illustrates that, with one exception, the respective samples clustered according to treatment, pairwise comparisons of these two groups revealed no significant differences in miRNA profiles (**Figure 4**). Notably, mmu-miR-3559-3p emerged as a regulated miRNA candidate in two different pairwise comparisons since its expression was altered in shock-fluoxetine mice in comparison to both shock-vehicle (**Figure 3A**) and no-shock-fluoxetine mice (**Figure 3B**).

Taken together, microarray analyses revealed that, in shocked mice, on day 74 after subjection of mice to footshock, the therapeutic effect of fluoxetine (**Figure 1**) went along with a significant decrease in prefrontal cortical rno-miR-3559-3p and mmu-miR-1971 expression as well as with a trend of reduction in prefrontal cortical ebv-miR-BART8-3p and mmu-miR-1947-3p expression (**Figure 3A**). Finally, our analyses revealed that none of the miRNA candidates tested was altered by traumatic stress *per se* (**Figure 2**) or by fluoxetine treatment *per se* (**Figure 4**) which suggests that fluoxetine treatment interacts with traumatic stress to alter the expression levels of the mentioned miRNA candidates.

RT-qPCR ANALYSIS CONFIRMED THAT FLUOXETINE TREATMENT ALTERS THE EXPRESSION OF mmu-miR-1971 AND mmu-miR-33-5p IN THE PFC OF SHOCKED MICE

Two out of the five array-identified miRNA candidates (rno-miR-3559-p, mmu-miR-1971, ebv-miR-BART8-3p, mmu-miR-1947-3p, mmu-miR-33-5p) could be validated by miRCURY LNA™ RT-qPCR: calculation of the statistical significance of RT-qPCR results with two-way ANOVA followed by Bonferroni *post hoc* correction confirmed a statistical trend toward a fluoxetine-mediated increase in prefrontal cortical mmu-miR-33-5p expression in shock-fluoxetine mice comparison to no-shock-fluoxetine mice (**Figure 5C**: Bonferroni posttest of shock-fluoxetine versus no-shock-fluoxetine: $t = 2.205$, $p = 0.055$). Most important, we observed a statistically significant reduction in mmu-miR-1971

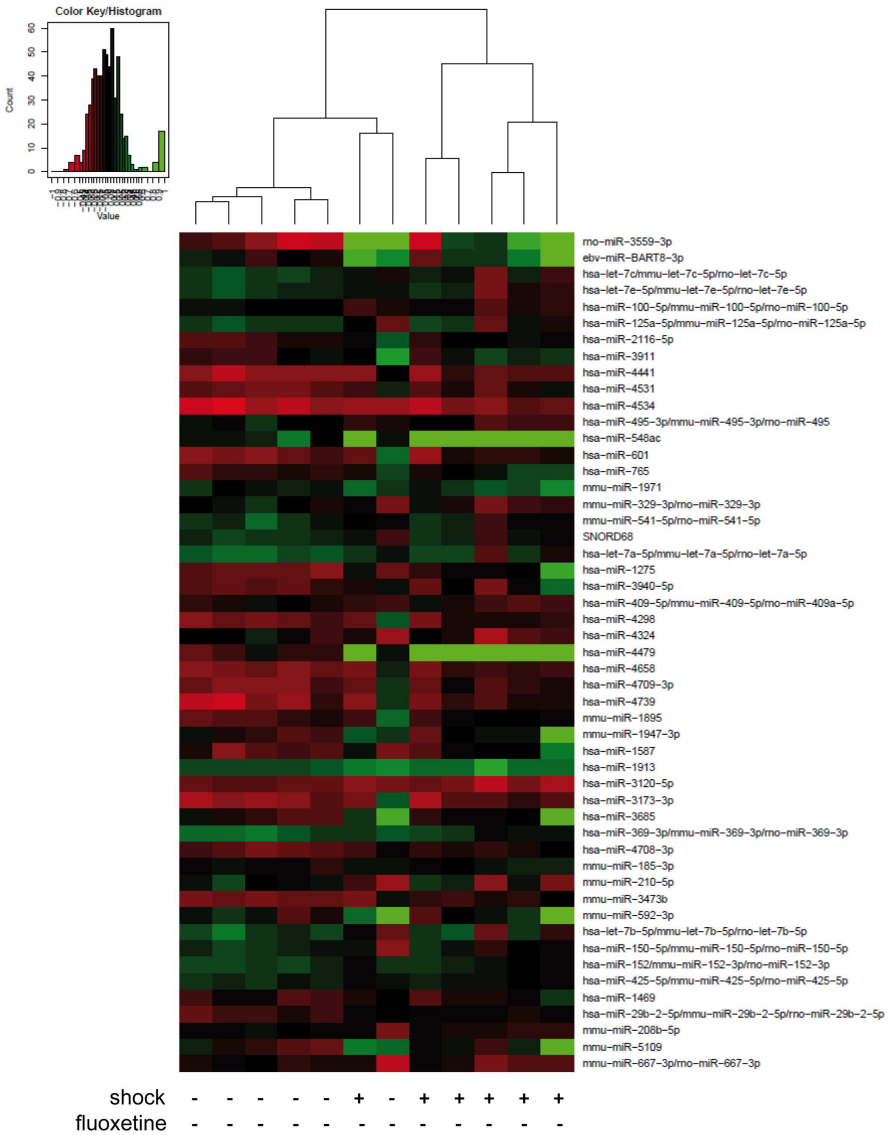

FIGURE 2 | Traumatic footshock does not significantly alter mouse PFC miRNA profiles in the long-term. Here, results of the hierarchical cluster analysis (HCA) of the footshocked vehicle-treated (shock-vehicle) versus the non-shocked vehicle-treated (no-shock-vehicle) samples are presented in a heatmap. The top 50 miRNA candidates with the lowest corrected *p*-values (resulting from pairwise comparison of the two groups shown here) were included. MiRNA expression levels were determined with LNA™ miRNA microarray analysis of mouse prefrontal cortex (PFC) total RNA samples. Samples were collected on day 74 after footshock ("shock") or mock treatment ("no-shock") from male C57/BL6/N mice ($n = 6$ per group). Each row represents a miRNA and each column represents a sample. Dendrograms represent complete-linkage clustering (by Euclidean distance) between samples. The sample clustering tree is shown on the top. The color scale illustrates the intensities of the relative miRNA expression levels: decreased scores are represented in red and increased in green, with intensity encoding magnitude. See Section "Materials and Methods" for statistical procedures. Vehicle, drinking water (solvent of fluoxetine).

expression in the PFC of shock-fluoxetine mice in comparison to shock-vehicle mice (**Figure 5A**: Bonferroni posttest of shock-vehicle versus shock-fluoxetine: $t = 2.509$, $p < 0.050$). RT-qPCR data do not allow the conclusion that fluoxetine *rescues* the footshock-induced increase in mmu-miR-1971 expression, since the latter failed to survive statistical correction (**Figure 5A**).

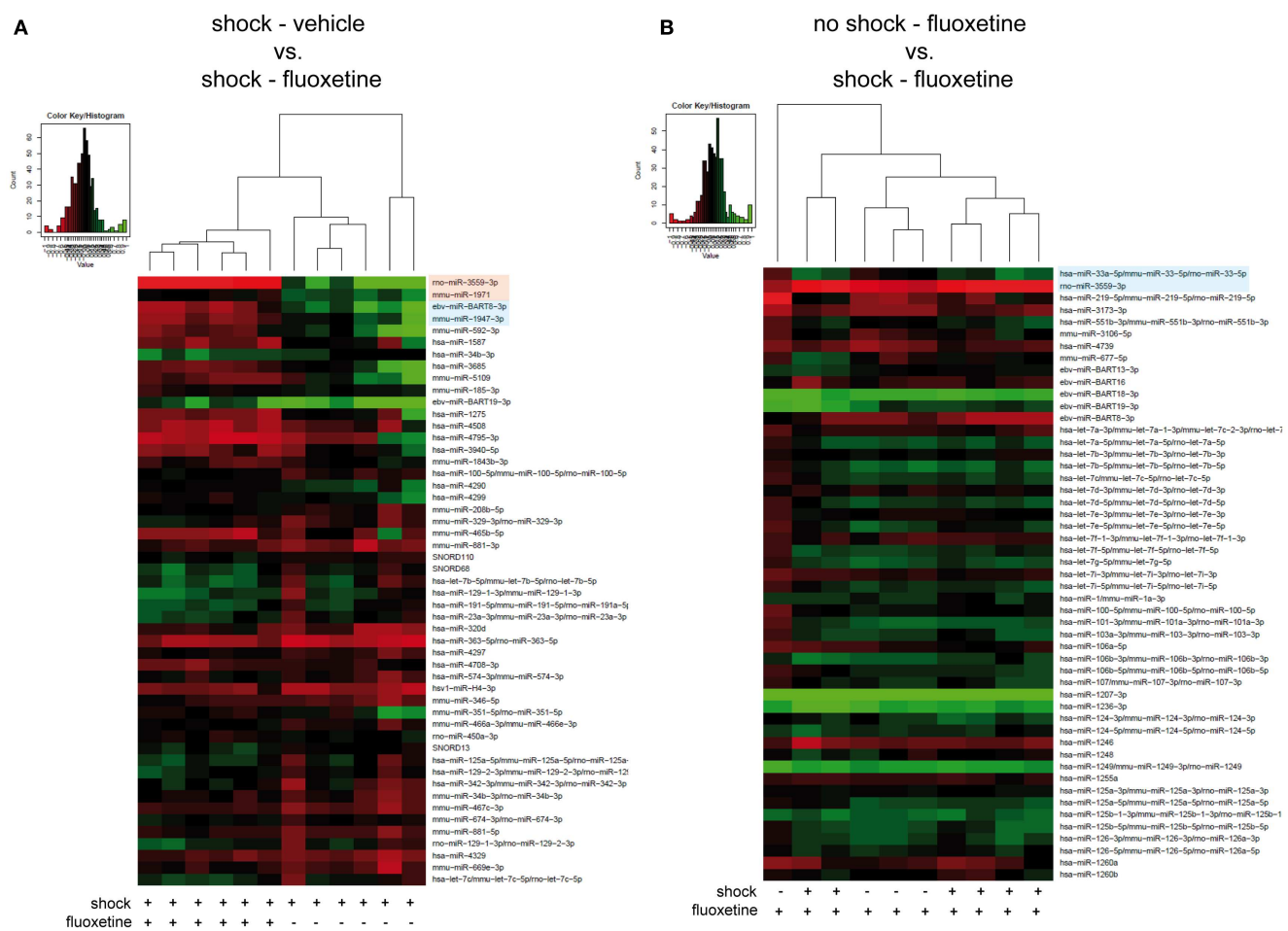

FIGURE 3 | Microarray analysis revealed fluoxetine treatment to significantly alter the expression of several miRNAs in the PFC of shocked mice. Here, results of the hierarchical cluster analysis (HCA) of **(A)** the footshocked vehicle-treated (shock-vehicle) versus the footshocked fluoxetine-treated (shock-fluoxetine) groups and of **(B)** the non-shocked fluoxetine-treated (no-shock-fluoxetine) versus the footshocked fluoxetine-treated (shock-fluoxetine) groups are presented in heatmaps. The top 50 miRNA candidates with the lowest corrected *p*-values [resulting from pairwise comparison of groups shown in **(A,B)**, respectively] were included. MiRNA expression levels were determined with LNA™ miRNA microarray analysis of mouse prefrontal cortex (PFC) samples. Samples were collected on day 74 after footshock ("shock") or mock treatment ("no-shock") of male C57/BL6/N mice. Each row represents a miRNA and each column represents a sample. Dendrograms represent complete-linkage clustering (by Euclidean distance) between samples. The sample clustering tree is shown on the top. The color scale illustrates the intensities of the relative miRNA expression levels: decreased scores are represented in red and increased in green, with intensity encoding magnitude. Significant alterations in miRNA expression levels determined by pairwise *t*-tests are highlighted in bright pink (corr. $p < 0.05$), statistical trends (corr. $p < 0.1$) in bright blue. Note that two samples of the no-shock-fluoxetine group were excluded during data processing (shock-vehicle: $n = 6$ per group; shock-fluoxetine: $n = 6$; no-shock-fluoxetine: $n = 4$). Statistical procedures are explained in the Section "Materials and Methods" and data are presented in the Section "Results" Vehicle, drinking water (solvent of fluoxetine).

Furthermore, despite a significant treatment effect in the two-way ANOVA ($F_{1,20\ \text{shock}} = 4.494$, $p = 0.030$), Bonferroni posttests did not detect any significant fluoxetine-mediated changes in relative expression of mmu-miR-1947-3p. Thus, we cannot consider mmu-miR-1947-3p as a fully validated candidate (**Figure 5B**). Finally, despite repetitive tries and employment of optimized LNA™ -technology based miRNA primer sets, expression of rno-miR-3559-3p and ebv-miR-BART8-3p could not be detected by RT-qPCR. Given that, we speculate that the array-detected rno-miR-3559-3p and ebv-miR-BART8-3p signals might possibly represent technical artifacts.

In summary, the most important conclusion of this study is that in the PTSD mouse model studied here, the therapeutic action of fluoxetine (**Figure 1**) is accompanied by a significant reduction in prefrontal cortical mmu-miR-1971 expression on day 74 after shock exposure (**Figures 3A** and **5B**).

miRNA TARGET PREDICTION AND GENE ONTOLOGY ANALYSIS

Finally, to get an idea of the potential role of mmu-miR-1971 and mmu-miR-33-5p in PTSD and of their general function, we performed an *in silico* analysis of target genes regulated by these two miRNA candidates: analysis performed with the

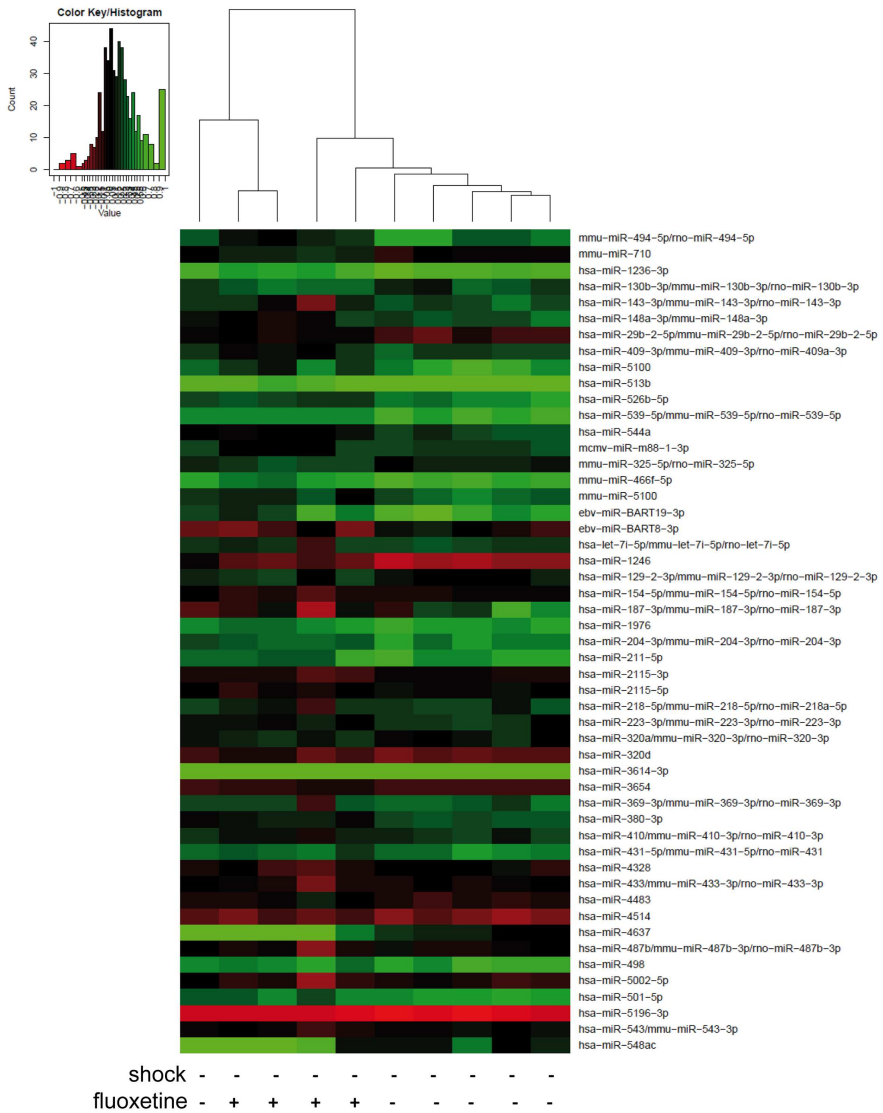

FIGURE 4 | In non-shocked mice, fluoxetine treatment does not significantly alter mouse PFC miRNA profiles in the long-term. Here, results of the hierarchical cluster analysis (HCA) of the non-shocked vehicle-treated (no-shock-vehicle) versus the non-shocked fluoxetine-treated (no-shock-fluoxetine) groups are presented in a heatmap. The top 50 miRNA candidates with the lowest corrected *p*-values (resulting from pairwise comparison of the two groups shown here) were included. MiRNA expression levels were determined with LNA™ miRNA microarray analysis of mouse prefrontal cortex (PFC) total RNA samples. Samples were collected on day 74 after footshock ("shock") or mock treatment ("no-shock") of male C57/BL6/N mice (no-shock-vehicle: $n = 6$ per group: no-shock-fluoxetine: $n = 4$). Each row represents a miRNA and each column represents a sample. Dendrograms represent complete-linkage clustering (by Euclidean distance) between samples. The sample clustering tree is shown on the top. The color scale illustrates the intensities of the relative miRNA expression levels: decreased scores are represented in red and increased in green, with intensity encoding magnitude. See Section "Materials and Methods" for statistical procedures. Vehicle, drinking water (solvent of fluoxetine).

miRWalk database[3] (52) revealed several validated target genes of mmu-miR-33-5p (**Table 3**), but none of mmu-miR-1971. Then, we used computational methods to predict potential target genes of mmu-miR-1971: we applied TargetScanMouse 6.2[4] (53) and MirTarget2[5] (54, 55); we included only those predicted target genes

[3]http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/mirnatargetpub.html

[4]http://www.targetscan.org/mmu_61/

[5]http://mirdb.org/miRDB/

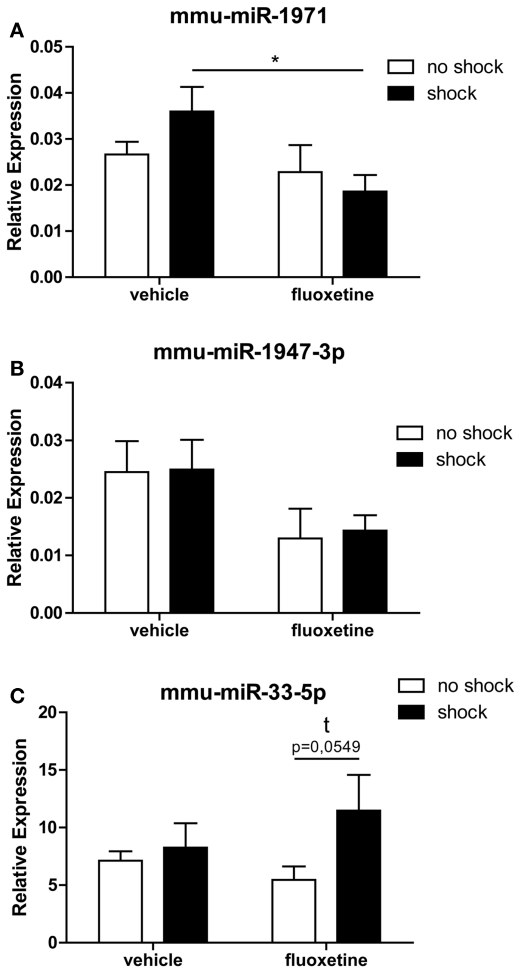

FIGURE 5 | RT-qPCR analysis confirmed that fluoxetine treatment alters the expression of mmu-miR-1971 and mmu-miR-33-5 in the PFC of shocked mice. Depicted are results of the RT-qPCR analysis of the relative expression levels of the candidate microRNAs mmu-miR-1971 **(A)**, mmu-miR-1947-3p **(B)**, and mmu-miR-33-5p **(C)** compared between the no-shock-vehicle, no-shock-fluoxetine, shock-vehicle, and shock-fluoxetine groups ($n = 6$ per group). Prefrontal cortex (PFC) samples employed for RT-qPCR analyses were identical to those used for microarray analyses. Mmu-miR-100-5p was used for normalization using the $\Delta\Delta C_t$ method. Presented data are means ± SEM. Statistical analysis was performed using two-way ANOVA and Bonferroni *post hoc* tests. Statistical significance of Bonferroni *post hoc* tests is indicated by $*p < 0.05$. See Section "Results" for statistical data. Vehicle, drinking water (solvent of fluoxetine).

that were identified with both approaches into subsequent GO analysis by employing GenericGeneOntologyTermFinder[6] (56) and REViGO[7] (57) (**Table 2**). The molecular functions of predicted mmu-miR-1971 target genes are mainly associated with small molecule and nucleic acid binding (**Table 2**). Moreover, most of them are involved in metabolic processes such as organic compound and RNA metabolism (**Table 2**).

[6]http://go.princeton.edu/cgi-bin/GOTermFinder
[7]http://revigo.irb.hr/

Table 2 | Gene ontology analysis of predicted mmu-miR-1971 target genes.

GO ID	GO term	Corr. *p*-value
GO MOLECULAR FUNCTION		
GO:0005488	Binding	8.98E−09
GO:0097159	Organic cyclic compound binding	2.97E−10
GO:0003723	RNA binding	6.98E−04
GO:0003676	Nucleic acid binding	2.54E−10
GO:0000166	Nucleotide binding	2.63E−04
GO:0036094	Small molecule binding	7.58E−04
GO:0043167	Ion binding	3.22E−05
GO:0005515	Protein binding	7.20E−05
GO:0003677	DNA binding	3.85E−05
GO BIOLOGICAL PROCESS		
GO:0006725	Cellular aromatic compound metabolic process	2.71E−06
GO:0008152	Metabolic process	2.50E−05
GO:0009987	Cellular process	1.02E−06
GO:0065007	Biological regulation	2.47E−06
GO:0006606	Protein import into nucleus	6.,19E−03
GO:0034654	Nucleobase-containing compound biosynthetic process	1.02E−05
GO:0046483	Heterocycle metabolic process	2.27E−06
GO:0044238	Primary metabolic process	6.15E−06
GO:0019438	Aromatic compound biosynthetic process	1.55E−05
GO:0071704	Organic substance metabolic process	3.73E−05
GO:0016071	mRNA metabolic process	7.31E−04
GO:0018130	Heterocycle biosynthetic process	1.65E−05
GO:0009058	Biosynthetic process	9.77E−05
GO:0000398	Nuclear mRNA splicing, via spliceosome	7.18E−04
GO:0044271	Cellular nitrogen compound biosynthetic process	2.77E−05
GO:0006807	Nitrogen compound metabolic process	8.77E−06
GO:0010468	Regulation of gene expression	4.09E−08
GO:0050794	Regulation of cellular process	1.33E−04
GO:0043170	Macromolecule metabolic process	1.36E−05
GO:0010467	Gene expression	5.25E−08
GO:0019222	Regulation of metabolic process	1.60E−04
GO:0044237	Cellular metabolic process	8.81E−06
GO:0016070	RNA metabolic process	8.04E−08
GO:0044260	Cellular macromolecule metabolic process	4.73E−06

Indicated are gene ontology IDs (GO ID), gene ontology terms (GO term), and corrected p-values as determined by GenericGeneOntologyTermFinder (http://go.princeton.edu/cgi-bin/GOTermFinder) and REViGO (http://revigo.irb.hr/).

Table 3 | Gene ontology analysis of validated mmu-miR-33-5p target genes.

GO ID	GO term	Annotated genes	Corr. *p*-value
GO MOLECULAR FUNCTION			
GO:0005488	Binding	Zp3, Lin28, Hprt1, Mos, H2afx, Ctdspl, H2afz, Fas, Rfpl4, Mt1, Ccnb2, Mbp, Dppa3, H1foo, Cd320, Dicer1, Hnt, Cpeb1, Srebf2, Ldlr, Cpt1a, Bmp4, Camk2g, Fgf21, Ccne1, Dnmt3b, Sycp3, Sirt6, Pou5f1, Abcg1	1.02E−06
GO:0035198	miRNA binding	Dicer1, Lin28, Pou5f1	8.49E−06
GO:0097159	Organic cyclic compound binding	H1foo, Cd320, Dicer1, Cpeb1, Srebf2, Lin28, Hprt1, Camk2g, Mos, H2afx, H2afz, Dnmt3b, Sycp3, Sirt6, Pou5f1, Abcg1	5.09E−03
GO:0005515	Protein binding	Zp3, Lin28, Hprt1, H2afx, H2afz, Fas, Rfpl4, Ccnb2, Mbp, Dppa3, Cd320, Dicer1, Cpeb1, Srebf2, Ldlr, Cpt1a, Bmp4, Camk2g, Fgf21, Ccne1, Dnmt3b, Sycp3, Abcg1, Pou5f1	1.02E−06
GO BIOLOGICAL PROCESS			
GO:0000003	Reproduction	H1foo, Dicer1, Zp3, Cpeb1, Lin28, Bmp4, Mos, H2afx, Ifitm3, Sycp3	5.11E−04
GO:0048610	Cellular process involved in reproduction	H1foo, Zp3, Cpeb1, Lin28, Sycp3, Bmp4, Mos, H2afx	4.52E−05
GO:0032502	Developmental process	Zp3, Lin28, Hprt1, H2afz, Fas, Ccnb2, Mbp, Dppa3, Hnt, Dicer1, Bmp4, Camk2g, Ccne1, Dnmt3b, Sycp3, Abcg1, Pou5f1	3.72E−03
GO:0042221	Response to chemical stimulus	Mbp, Dicer1, Srebf2, Lin28, Hprt1, Bmp4, Fgf21, Ifitm3, Fas, Dnmt3b, Mt1, Abcg1, Pou5f1	2.83E−03
GO:0071840	Cellular component organization or biogenesis	Hprt1, H2afx, H2afz, Fas, Ccnb2, Mbp, H1foo, Dppa3, Hnt, Dicer1, Cpeb1, Cpt1a, Bmp4, Dnmt3b, Sycp3, Abcg1, Pou5f1, Sirt6	1.33E−04
GO:0003133	Endodermal-mesodermal cell signaling	Bmp4, Pou5f1	3.54E−03
GO:0006325	Chromatin organization	Dppa3, H1foo, H2afx, H2afz, Sycp3, Dnmt3b, Pou5f1, Sirt6	1.36E−04
GO:0006259	DNA metabolic process	Dppa3, H1foo, Bmp4, H2afx, Ccne1, H2afz, Sycp3, Dnmt3b, Sirt6	2.32E−04
GO:0022402	Cell cycle process	H1foo, Dicer1, Cpeb1, Camk2g, Bmp4, Mos, H2afx, Sycp3, Ccnb2	5.36E−04
GO:0045595	Regulation of cell differentiation	Mbp, Dicer1, Hnt, Lin28, Bmp4, Ccne1, Fas, Dnmt3b, Pou5f1, Abcg1	6.50E−04
GO:0007049	Cell cycle	H1foo, Dicer1, Cpeb1, Camk2g, Bmp4, Mos, H2afx, Ccne1, Sycp3, Ccnb2	1.05E−03
GO:0006807	Nitrogen compound metabolic process	Zp3, Lin28, Hprt1, H2afx, H2afz, H1foo, Dppa3, Dicer1, Srebf2, Cpeb1, Cpt1a, Ldlr, Bmp4, Ccne1, Dnmt3b, Sycp3, Abcg1, Pou5f1, Sirt6	1.44E−03
GO:0040029	Regulation of gene expression, epigenetic	Dppa3, Dicer1, Dnmt3b, Lin28, Pou5f1	1.29E−03
GO:0048519	Negative regulation of biological process	Mbp, Dppa3, Dicer1, Zp3, Hnt, Srebf2, Lin28, Bmp4, Ifitm3, Fas, Dnmt3b, Mt1, Sycp3, Pou5f1, Abcg1	1.78E−03
GO:0045834	Positive regulation of lipid metabolic process	Zp3, Ldlr, Cpt1a, Abcg1	7.65E−03
GO:0016458	Gene silencing	Dicer1, Dnmt3b, Lin28, Pou5f1	7.65E−03
GO:0010033	Response to organic substance	Dicer1, Srebf2, Lin28, Hprt1, Bmp4, Fgf21, Ifitm3, Fas, Dnmt3b, Abcg1, Pou5f1	4.09E−03
GO:0050794	Regulation of cellular process	Zp3, Lin28, Hprt1, Mos, Ifitm3, Fas, Mt1, Ccnb2, Mbp, Dppa3, Hnt, Dicer1, Cpeb1, Srebf2, Ldlr, Cpt1a, Bmp4, Fgf21, Ccne1, Dnmt3b, Sycp3, Sirt6, Abcg1, Pou5f1	4.26E−03

(Continued)

Table 3 | Continued

GO ID	GO term	Annotated genes	Corr. *p*-value
GO:0071824	Protein-DNA complex subunit organization	H1foo, H2afz, Sycp3, H2afx	5.38E−03
GO:0080090	Regulation of primary metabolic process	Dppa3, Dicer1, Zp3, Cpeb1, Srebf2, Ldlr, Cpt1a, Lin28, Hprt1, Bmp4, Ccne1, Dnmt3b, Sirt6, Pou5f1, Ccnb2, Abcg1	7.43E−03
GO:0006323	DNA packaging	H1foo, H2afz, Sycp3, H2afx	7.37E−03
GO:0003130	BMP signaling pathway involved in heart induction	Bmp4, Pou5f1	3.54E−03
GO:0034641	Cellular nitrogen compound metabolic process	Zp3, Lin28, Hprt1, H2afx, H2afz, H1foo, Dppa3, Dicer1, Srebf2, Cpeb1, Cpt1a, Ldlr, Bmp4, Ccne1, Dnmt3b, Sycp3, Abcg1, Pou5f1, Sirt6	5.92E−04
GO:0050793	Regulation of developmental process	Mbp, Dicer1, Zp3, Hnt, Lin28, Bmp4, Ccne1, Fas, Dnmt3b, Pou5f1, Abcg1	1.71E−03
GO:0048523	Negative regulation of cellular process	Mbp, Dppa3, Dicer1, Zp3, Hnt, Srebf2, Lin28, Bmp4, Ifitm3, Fas, Dnmt3b, Mt1, Sycp3, Pou5f1, Abcg1	4.54E−04

Indicated are gene ontology IDs (GO ID), gene ontology terms (GO term), the annotated gene names, and corrected p-values as determined by GenericGeneOntologyTermFinder (http:// go.princeton.edu/ cgi-bin/ GOTermFinder) and REViGO (http:// revigo.irb.hr/).

Then, to characterize possible molecular functions of validated mmu-miR-33-5p regulated targets, we performed GO analysis as described above. We found that most of the validated mmu-miR-33-5p targets are associated with protein, miRNA, and organic compound binding (**Table 3**). Furthermore, many of the validated mmu-miR-33-5p target genes are involved in cellular and developmental processes, and most important, in epigenetic regulation of gene expression and lipid metabolic processes (**Table 3**).

Taken together, target prediction and GO analyses revealed several predicted target genes of mmu-miR-1971 and several validated target genes mmu-miR-33-5p which might possibly be involved *inter alia* in PTSD pathobiology or fluoxetine-mediated alterations of molecular pathways. Interestingly, amongst these target genes we found none which had previously been repetitively associated with PTSD, like for instance FKBP5 (8), CDK5 (58), or synapsin (28).

DISCUSSION

Here, we present the first study exploring miRNA expression profiles in a PTSD mouse model. In summary, we demonstrate that the therapeutic action of fluoxetine in shocked mice (**Figure 1**) is correlated with a significant reduction in prefrontal cortical mmu-miR-1971 expression levels on day 74 after shock exposure (**Figures 1** and **5A**). The significance of this finding is supported by results of the unsupervised HCA of the shock-vehicle versus the shock-fluoxetine group which revealed that samples of both groups clustered perfectly according to treatment (**Figure 3A**) thereby demonstrating that the miRNome is a factor that contributes to biological differences in these two groups. RT-qPCR data do not allow the conclusion that fluoxetine *rescues* the footshock-induced increase in mmu-miR-1971 expression, since the latter failed to survive correction for multiple testing (**Figure 5A**). Furthermore, our analyses revealed a trend toward an increase of prefrontal cortical mmu-miR-33-5p expression in shock-fluoxetine mice in comparison to no-shock-fluoxetine mice (**Figure 5C**). Interestingly, we found that traumatic stress *per se* and fluoxetine treatment *per se* did not lead to significant alterations of mouse miRNA profiles on day 74 after trauma exposure (**Figures 2** and **4**) which suggests that fluoxetine interacts with traumatic stress to alter expression levels of mmu-miR-1971 and mmu-miR-33-5p (**Figures 5A,C**). To the best of our knowledge, these two miRNA candidates have not been associated with psychiatric disorders so far. MiR-1971 has hitherto not even been associated with the central nervous system (CNS). Instead, in the only study reporting expression level changes of miR-1971 demonstrated that, in the bone marrow, miR-1971 was differentially expressed between patients suffering from acute lymphoblastic leukemia (ALL) and healthy donors (59). However, to our knowledge, the in that study newly identified miRNA sequence, which was termed hsa-miR-1971 thereby representing it as the human homolog of murine mmu-miR-1971, is not annotated in miRBase 19.0. Our miRNA target prediction and GO analysis revealed that for miR-1971 no target genes have been validated so far (**Table 2**). Hence, regulation and function of miR-1971 are largely unexplored yet and await further studies. However, GO analysis of predicted miR-1971 target genes allude that this above-average small (18nt) miRNA candidate might be involved *inter alia* in basic metabolic processes like heterocycle and organic substance metabolism (**Table 2**: $p = 2.27E{-}06$ and $p = 3.73E{-}05$, respectively); neurotransmitters like serotonin or modulators of the serotonergic tone might belong to the organic substances whose metabolism is targeted by miR-1971, but, however, our GO analysis provided no direct hint for this speculation.

In contrast, miR-33 has been studied more intensely. A fundamental biological role of miR-33-5p (previous miRBase ID: miR-33) is suggested by the fact that, according to miRBase 18.0, its sequence is highly conserved in human, mouse, and rat. MiR-33 was found to be downregulated in the hippocampus of rats with status epilepticus (60), to regulate the cell cycle (61, 62), to be associated with mouse atherosclerosis (63, 64), as well as with metabolism of cholesterol (65, 66). The latter finding is supported by our GO analysis which revealed the biological process termed "positive regulation of lipid metabolic processes" to be significantly enriched among validated mmu-miR-33-5p target genes (**Table 3**: $p = 7.65\text{E}{-}03$). Low blood levels of cholesterol were found to be associated with suicidality (67, 68), while PTSD patients were repeatedly reported to exhibit elevated cholesterol blood levels (69, 70). Cholesterol biosynthesis in glial cells was shown to be influenced by fluoxetine and other antidepressants (59). In turn, most interestingly, there is strong evidence for an influence of the cholesterol metabolism on fluoxetine treatment response both in rodents (60) and in humans (61). The synopsis of these findings fuels the speculation that in the PTSD mouse model studied here, the shock × fluoxetine interaction-mediated increase of prefrontal cortical mmu-miR-33-5p expression (**Figures 3B** and **5C**) might contribute to the previously reported influence of cholesterol metabolism on the response to fluoxetine treatment (59, 60). This synoptic speculation is worth addressing in future studies. However, even though our analyses do support an involvement of mmu-miR-33-5p in fluoxetine-mediated processes, they do neither speak for nor against the involvement of mmu-miR-33-5p in the *therapeutic* action of fluoxetine since we did *not* find significant mmu-miR-33-5p expression level differences between vehicle-treated and fluoxetine-treated shocked mice. Instead, our data suggest an involvement of mmu-miR-1971 in the therapeutic action of fluoxetine in footshocked mice.

Interestingly, fluoxetine was shown previously to regulate miRNA expression, namely to alter the expression of miR-16 in serotonergic raphe nuclei (71). However, in the here presented study which analyzed miRNA expression levels in another brain region, i.e., the PFC, in a different experimental context, miR-16 expression was not significantly altered by fluoxetine treatment. Since changes in miRNA expression can occur rapidly (72), we suppose that both traumatic stress and fluoxetine treatment might exert even stronger effects on miRNA expression at earlier time-points after challenge. At the late posttrauma time-point tested here, the *consequences* of trauma-stress and fluoxetine-mediated alterations of miRNA expression probably dominate alterations in miRNA expression itself. Moreover, it would also be interesting to evaluate miRNA profiles in other brain regions associated with PTSD, like for instance the hippocampus and the amygdala (31). To check for the generalizability of the results presented here, our findings should be validated in another mouse cohort and in other animal models for PTSD. Most important, it also remains to be tested, for instance by employing an *in vivo* knockdown approach, whether the miRNA candidates identified here *causally* contribute to the therapeutic effects of fluoxetine or not.

All in all, this study is the first that examined miRNA profiles in connection with PTSD and represents a promising starting point for further evaluation of the role of miRNAs in PTSD pathobiology.

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