MOLECULAR MECHANISMS IN STRESS AND TRAUMA RELATED DISORDERS

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MOLECULAR MECHANISMS IN STRESS AND TRAUMA RELATED DISORDERS

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Table of Contents

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EDITORIAL
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Editorial: Molecular Mechanisms in Stress and Trauma Related Disorders

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Keywords: autophagy, circadian biology, epigenetics, inflammation, major depression, posttraumatic stress disorder, psychosocial stress

Editorial on the Research Topic

Molecular Mechanisms in Stress and Trauma Related Disorders

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Psychosocial stress is ubiquitous in modern societies and represents a potentially preventable risk factor for a host of aberrant phenotypes (1). Interestingly, despite their ubiquity, psychosocial stressors can elicit diverse and often unpredictable psychologic and somatic outcomes among exposed individuals. What constellation of mechanisms determines whether some individuals will suffer a variety of negative consequences in the face of adverse environments, whereas others will remain intact or even thrive during periods of stress exposure? A body of evidence has suggested that this interindividual variability may result from complex molecular mechanisms that collectively shape individual responses and outcomes to stress at the cellular, physiological, neuroendocrine, and behavioral levels. However, the exact molecular contributors to such responses and outcomes are poorly understood. In this Research Topic, we present a series of both original studies and review articles that shed new light into how stress-related conditions may be influenced by processes such as inflammation, circadian biology, intracellular signaling pathways, oxidative stress, and epigenetics.

Stress exposure can trigger episodes of major depression (2). Furthermore, both MDD and childhood maltreatment have been previously shown to promote inflammation in a potentially synergistic manner (3), yet the underlying mechanisms are still incompletely understood. In an original study, de Punder et al. demonstrate that only patients with both depression and a history of childhood adversity exhibit heightened inflammation, whereas depressed subjects without childhood adversity have inflammatory profiles similar to those of control subjects. In a systematic review and meta-analysis, Perrin et al. show that increased inflammation in depression is associated with glucocorticoid resistance, primarily indicated by higher levels and impaired suppression of the stress hormone cortisol. Together these findings suggest the existence of

inflammatory subtypes of major depression that may be characterized by previous exposure to childhood adversity and/or aberrant glucocorticoid signaling.

Another key molecular process addressed herein is circadian biology and its particular involvement in trauma-related disorders. In an original study, Linnstaedt et al. show that genetic polymorphisms in circadian pathway genes influence risk for the development of posttraumatic stress symptoms following exposure to multiple types of psychologic trauma, including motor vehicle accidents, sexual assault, and burn injury. Complementarily, Agorastos et al. review how the stress system interacts at multiple levels with circadian biology and the potential relevance of these interactions for posttraumatic stress disorder. These articles underscore the importance of further dissecting the role of circadian biology in stress and trauma-related disorders.

Three original articles address how chemical modifications of nucleic acids, acting at multiple levels to influence DNA and RNA integrity and function, may be implicated in stress-related disorders. He et al. examine DNA methylation—one of the critical epigenetic mechanisms that regulate gene expression—and show that childhood maltreatment in humans is associated with higher, whereas bipolar disorder with lower, blood methylation levels of the gene expressing the cytokine and stem cell factor KIT ligand. Dick et al. employ a mouse model of chronic social defeat stress and show that chronic stress can set into motion adenosine-to-inosine RNA editing, a co-/posttranscriptional RNA modification that influences protein isoform expression, within the corticolimbic regions of the mouse brain). Boeck et al. examine oxidative stress and DNA damage in *postpartum* women and show that exposure to childhood maltreatment is positively associated with levels of 8-isoprostane, a marker of lipid peroxidation, but not with markers of oxidative DNA or RNA damage. Further studies will be needed to uncover how stress and trauma exposure may induce such chemical modifications of DNA and RNA to contribute to diverse health and disease outcomes.

Lastly, a series of review articles highlight novel roles for other signaling pathways and molecular processes. Gassen and Rein discuss the role of autophagy—an evolutionarily conserved intracellular pathway responsible for energy, organelle, and protein homeostasis—in depressive phenotypes and its potential involvement in antidepressant action. Stepan et al. discuss recent evidence suggesting a role for Hippo signaling—a signaling pathway involved in organ development, tissue homeostasis, and regeneration—in neuroplasticity and stress-related phenotypes. Gold and Kadriu provide a perspective on the physiologic and molecular mechanisms underlying the involvement of the lateral habenula—a brain region with antireward properties and bidirectional connections to the stress system—in the development of anhedonia and other depressive phenotypes. Papadopoulou et al. review how early life stressors—including those occurring before conception, *in utero*, and postnatally—get embedded to shape subsequent responses to stressful environments, and how this embedding takes place at the molecular level. By covering such diverse mechanisms and processes, these review articles underscore how several molecular and cellular mechanisms may interact at multiple levels and in complex ways to contribute to diverse outcomes after stress and trauma exposure.

In conclusion, this Research Topic has aimed at providing up-to-date evidence on the molecular mechanisms that may underlie the development of stress and trauma-related disorders. A wide range of molecular processes were examined, including inflammatory processes, circadian biology, key intracellular pathways such as autophagy and Hippo signaling, oxidative stress, and epigenetic regulation. It is our hope that this compilation and the resultant discussion will stimulate fruitful research that aims at unraveling the molecular cascade of events through which stressful experiences may contribute to the development of aberrant disease phenotypes. Furthermore, while keeping in mind that these mechanisms likely act complementarily and in complex ways to shape diverse stress-related outcomes across individuals, deeper insights into the role of individual molecular mechanisms can have important implications for identifying novel molecular targets, eventually enhancing our ability to predict, prevent, diagnose, and treat stress-related disorders.

AUTHOR CONTRIBUTIONS

AZ wrote the first draft of the manuscript. MS and GC critically revised the manuscript. All authors read and approved the submitted version.

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ORIGINAL RESEARCH
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Evaluation of the Association Between Genetic Variants in Circadian Rhythm Genes and Posttraumatic Stress Symptoms Identifies a Potential Functional Allele in the Transcription Factor *TEF*

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Previous studies suggest that genetic variants within genes affecting the circadian rhythm influence the development of posttraumatic stress symptoms (PTSS). In the present study, we used data from three emergency care-based cohorts to search genetic variants in circadian pathway genes previously associated with neuropsychiatric disorders for variants that influence PTSS severity. The three cohorts used included a discovery cohort of African American men and women enrolled following motor vehicle collision ($n = 907$) and two replication cohorts: one of multi-ethnic women enrolled following sexual assault ($n = 274$) and one of multi-ethnic men and women enrolled following major thermal burn injury ($n = 68$). DNA and RNA were collected from trauma survivors at the time of initial assessment. Validated questionnaires were used to assess peritraumatic distress severity and to assess PTSS severity 6 weeks, 6 months, and 1 year following trauma exposure. Thirty-one genetic variants from circadian rhythm genes were selected for analyses, and main effect and potential gene*stress and gene*sex interactions were evaluated. Secondary analyses assessed whether associated genetic variants affected mRNA expression levels. We found that six genetic variants across five circadian rhythm-associated genes predicted PTSS outcomes following motor vehicle collision

($p < 0.05$), but only two of these variants survived adjustment for multiple comparisons (False Discovery Rate < 5%). The strongest of these associations, an interaction between the PAR-zip transcription factor, thyrotroph embryonic factor (*TEF*) variant rs5758324 and peritraumatic distress, predicted PTSS development in all three cohorts. Further analysis of genetic variants in the genetic region surrounding *TEF*rs5758324 (±125,000 nucleotides) indicated that this allele showed the strongest association. Further, *TEF* RNA expression levels (determined via RNA-seq) were positively associated with PTSS severity in distressed individuals with at least one copy of the *TEF*rs5758324 minor allele. These results suggest that rs5758324 genetic variant in *TEF*, a regulator of clock-controlled genes and key mediator of the core circadian rhythm, influence PTSS severity in a stress-dependent manner.

Keywords: PTSD, genetic polymorphism, circadian rhythm, trauma, TEF, RNA

INTRODUCTION

Unfortunately, traumatic events are common in life. For instance, each year in the United States, more than 11 million individuals experience a motor vehicle collision (MVC) (1), more than one million women are sexually assaulted (2), and more than 50,000 individuals are hospitalized after major thermal burn injury (3). Although most individuals recover following trauma exposure, a substantial proportion develop adverse post-traumatic neuropsychiatric sequelae such as persistent posttraumatic stress symptoms (PTSS).

Individual genetic differences influence vulnerability to PTSS following trauma exposure: data from twin research and genetic association studies estimate the heritability of PTSS to be between 29 and 40 percent (4–6). The study of genetic variants associated with vulnerability to PTSS has provided a number of valuable clues and new directions in understanding the pathogenesis and manifestations of the disorder. The first GWAS for PTSS identified an association between genetic variants in the retinoid-related orphan receptor alpha gene, a gene that stabilizes environmental influences on the circadian rhythm (7), and PTSS vulnerability (8). This association has been subsequently replicated in both studies of PTSS (9, 10) and other stress-related disorders (11–17) [but also failed to replicate for PTSS (18)].

In addition to these data, evidence continues to accrue more broadly that the circadian rhythm plays an important role in PTSS development and symptom expression (19–30). The circadian rhythm entrains the body to 24 h light-dark cycles through transcription-translation feedback loops in the hypothalamic suprachiasmatic nucleus and throughout the body in cells of almost every tissue (31, 32). This cycling allows the body to adjust behavior, metabolism, and physiology in response to environmental cues. One specific type of environmental stimulus known to influence the circadian rhythm (and vice versa) is physiological stress exposure (33, 34). It has been hypothesized that interactions between circadian and stress systems may contribute to the underlying pathogenesis of neuropsychiatric disorders (35), and a growing body of evidence supports this hypothesis (36–40).

Evidence supporting the interplay between the circadian clock and stress systems includes data from a variety of studies. For instance, clock genes (e.g., *PER1, PER2*, *TIMELESS*, *NPAS2*) have been shown to be regulated by stress hormones such as glucocorticoids (GC) (41–43). GC release throughout a 24 h period follows a circadian rhythm in which levels are highest at the beginning of the wake period and lowest at the beginning of the sleep period (44). Animals with key circadian rhythm genes knocked out have altered GC levels (45). Additionally, FKBP5, a chaperone protein that plays a role in the regulation of circulating GC levels, and has been shown to play a role in stress related disorders (46, 47), is rhythmically expressed in most tissues (48).

In the current study, we used prospective data from three emergency department based trauma cohorts (one discovery and two replication cohorts) to test the hypothesis that genetic variants in circadian rhythm genes predict PTSS development following trauma exposure. In addition, we hypothesized that the relationship between polymorphisms in circadian rhythm genes and PTSS might be dependent on stress levels (as measured via reported peritraumatic distress levels or via the critical stress regulator *FKBP5*). We found a significant, stress-dependent relationship between a genetic variant in the circadian rhythm-associated thyrotroph embryonic factor (*TEF*) gene, rs5758324, and PTSS development that replicated across all three cohorts. In addition, analyses of mRNA expression data from the discovery cohort suggest that this allele is functional.

METHODS

Study Design, Setting, and Eligibility Criteria

Motor Vehicle Collision (MVC) Study

This prospective longitudinal study enrolled African American individuals ≥18 and ≤65 years of age presenting to the ED within 24 h of MVC. The details of this study have been described

Abbreviations: VC, motor vehicle collision; SA, sexual assault; MThBI, major thermal burn injury; ED, emergency department; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; *TEF*, thyrotroph embryonic factor; PTSS, posttraumatic stress symptoms; GWAS, genome wide association study.

previously (49). This study was approved by the institutional review boards at the data-coordinating center (The University of North Carolina at Chapel Hill) and at all participating hospitals. Each study participant provided written informed consent before enrollment.

Eligible and consenting participants provided blood samples in the ED and completed an ED interview evaluation. Research assistants performed interview evaluations at the time of the ED visit using a web-based survey with explicit definitions of variables. Patients who were not alert and oriented were excluded, as were patients who did not self-identify as African American, pregnant patients, prisoners, patients unable to read and understand English, or patients taking opioids above a total daily dose of 30 mg of oral morphine or equivalent. Data extraction from the ED medical record obtained injury characteristics and medications administered in the ED. Participant demographic characteristics (including age, sex, and educational attainment) were obtained from the ED medical record and from participant self-report. Six weeks, six months, and one year after the MVC, participants completed a follow-up interview by telephone, online, or via mail.

Assessments and outcome definitions for the MVC study

MVC-related PTSS was assessed at all three follow-up timepoints (6 weeks, 6 months, and 1 year) using the Impact of Event Scale: Revised (IESR) (50). This 22-item questionnaire includes avoidance, intrusion and hyperarousal subscales. Scores range from 0 to 88. Using a previously validated cutoff [IESR $\geq$ 33 (51)], we estimated that 29% of individuals enrolled following MVC had PTSD 6 months following trauma exposure.

Peritraumatic distress in the ED was measured using the Peritraumatic Distress Inventory, a 13-item questionnaire assessing the level of distress experienced immediately after a traumatic event (52). This assessment measures life threat, loss of control, helplessness/anger, and guilt/shame. Each item on the questionnaire was evaluated via numeric rating scale from 0 (no distress) to 4 (high distress). A validated cut-off score of 23 was used to identify those with substantial distress (53).

Sexual Assault (SA) Study

This prospective longitudinal study is similar in design to its pilot study described previously (54) and to the MVC study described above. Women $\geq$18 and $\leq$65 years of age presenting to one of 13 sexual assault nurse examiner (SANE) programs within 72 h of sexual assault trauma were enrolled. Women unable to give informed consent (e.g., due to intoxication) were excluded, as were women who were hospitalized after sexual assault, lived with their assailant, were prisoners, were pregnant, did not have a telephone, and/or did not live within driving distance for follow-up interviews. Institutional Review Board (IRB) approval was obtained at all study sites, and all study participants provided written informed consent.

Assessments and outcome definitions for the SA study

SA-related PTSS was assessed at three follow-up timepoints (6 weeks, 6 months, and 1 year) using the civilian version of the posttraumatic stress disorder checklist (PCL) (55). This validated 17-item questionnaire assesses posttraumatic distress symptoms in relation to a stressful experience. A total symptom severity score (range = 17–85) can be obtained by summing the scores from each of the 17 items that have response options ranging from 1 "Not at all" to 5 "Extremely." Using this PCL scale, we estimated the incidence of PTSD 6 months following trauma exposure in this cohort of sexual assault survivors to be 62%.

One week following sexual assault, participants were asked about peritraumatic distress symptoms in the period since the assault using measures from PROMIS 8b. These measures include statements such as "I felt fearful," "I felt nervous," "I felt anxious," "I felt tense," and "I felt uneasy." Participants rated each item with a score from 1 to 5 (ranging from never to always), with a total possible score from 8 to 40. The median score was used to distinguish women with high distress from those with low distress.

Major Thermal Burn Injury (MThBI) Study

Patients undergoing tissue autograft after MThBI between February 2012 through June 2015 at one of the three burn centers (University of North Carolina, Chapel Hill, NC, MedStar Washington Hospital Center, Washington, DC, and University of South Florida, Tampa, FL) were enrolled. Exclusion criteria included age <18 or >65, admission >72 h after MThBI, estimated total body surface area (TBSA) burn >30%, intentional, electrical or a chemical mechanism, autograft performed >14 days after admission to burn center or autograft decision made >7 days after admission, Childs-Pugh liver failure stage B or C, end stage renal disease, chronic opioid use >20 morphine equivalents per day before burn, preburn skin disorder causing pruritus, substantial co-morbid injury (e.g., blast injury resulting in major trauma in addition to burn), pregnancy or breastfeeding, residing greater than 100 miles from site, and burn that required escharotomy. In addition, individuals unwilling to provide a blood sample, prisoners, suicidal, homicidal, psychotic individuals, and individuals who did not read and speak English were excluded. The Institutional Review Board at each burn center approved the study protocol, and each participant provided written informed consent.

Assessments and outcome definitions for the MThBI study

MThBI-related PTSS severity was assessed 1 day, 6 weeks, 6 months, and 1 year following burn injury using the PCL questionnaire (as described above for the SA study). In this MThBI cohort, an estimated 13% of individuals reported PTSD 6 months following trauma exposure. This questionnaire was also administered in the immediate aftermath of trauma (day 1) and served as the measure for peritraumatic distress. A previously reported cutoff of 23 was used to distinguish individuals with high distress vs. those with low distress (56).

DNA Collection and Genotyping

Study personnel collected blood samples at the time of enrollment using PAXgene DNA tubes. Following DNA purification (PAXgene blood DNA kit, QIAGEN), genotyping using the Infinium Multi-Ethnic Global Array (MEGA, Illumina) was performed (AKESOgen, Inc; Atlanta, GA). DNA from an

individual with known genotype (NA19819, Coriell Institute, Camden, NJ) and two repeat samples were included in each genotyping batch (96 samples) to ensure genotypic accuracy and reliability.

Thirty-one genetic variants across nine circadian rhythm associated genes were selected for analyses based on previous association with PTSS or related neuropsychiatric disorders (see **Table 1** for references) and whether they were included on the MEGA chip. These genetic variants had excellent call rates (>98%) and were in Hardy-Weinberg equilibrium (**Supplementary Table 1**, $p \leq 0.05$).

RNA Collection and Sequencing

PAXgene RNA tubes were used to collect blood in the ED at the time of enrollment. Total RNA was isolated using the PAXgene blood miRNA kit (QIAGEN) and stored at −80°C until use. RNA concentration and purity were measured using a NanoDrop 1000 (Nanodrop Technologies, Wilmington, DE).

One hundred and eighty-four samples were selected for RNA sequencing based on whether individuals reported relatively high or relatively low levels of PTSS over time after MVC. This selection occurred intermittently throughout the study and well prior to the planning of these specific analyses.

Template libraries for total RNA sequencing were produced from 600 ng total RNA using Ovation Human Blood RNA-Seq Library Systems kit (NuGen, San Carlos, CA) according to manufacturer's specifications. Libraries were multiplexed in groups of six and sequenced on a HiSeq 2500 at the University of North Carolina at Chapel Hill High Throughput Sequencing Facility. Raw sequencing reads were aligned to the human hg19 genome assembly using STAR (version 2.4.2a) (57). Expression levels of each transcript (n = 20,353) were estimated via RSEM (58) using University of California Santa Cruz (UCSC) known gene transcript and gene definitions. Raw RSEM read counts for all samples were normalized to the overall upper quartile (59) before comparison and visualization. Consistent with study goals, only messenger RNA aligning to the *TEF* gene was included in these analyses.

TABLE 1 | Baseline characteristics of study participants.

	Discovery	Replication cohorts	
Characteristic	**MVC[a]**	**SA[b]**	**MThBI[c]**
Enrolled, n	930	274	68
Age, years, mean (SD)	35.1 (12.7)	28.8 (11.4)	37.6 (12.2)
Females, *n* (%)	578 (62.2)	274 (100)	18 (26.5)
African American, *n* (%)	930 (100)	46 (16.8)	27 (39.7)
Education, *n* (%)			
8–11 yrs	71 (7.6)	17 (6.2)	8 (11.8)
High school	290 (31.2)	70 (25.5)	24 (35.3)
Post-high school	42 (4.5)	12 (4.4)	2 (2.9)
Some college	338 (36.3)	124 (45.3)	23 (33.8)
College graduate	137 (14.7)	44 (16.1)	8 (11.8)
Post-graduate studies	36 (3.9)	7 (2.6)	3 (4.4)

[a]*MVC, motor vehicle collision cohort;* [b]*SA, sexual assault cohort;* [c]*MThBI, major thermal burn injury cohort.*

Selection of Circadian Rhythm Associated Genetic Variants for Primary Analyses

The genetic variants selected for analyses in this study originate from genes in the core circadian rhythm pathway that are involved directly in the core feedback mechanism or are transcriptional regulators of clock-controlled genes that affect tissue specific physiological processes such as neurotransmission, immune processes, and endocrine signaling (**Supplementary Figure 1**). We used a structured literature review to identify genetic variants within such circadian rhythm genes that have previously been shown to be associated with PTSS or related neuropsychiatric disorders. To perform this literature review, we first searched the PubMed (NCBI) database using the following search terms: "post-traumatic stress disorder"/"PTSD" and "*CLOCK*" or "*BMAL1*" or "*BMAL2*" or "*ARNTL*" or "*ARNTL2*" or "*PER1*" or "*PER2*" or "*PER3*" or "*CRY1*" or "*CRY2*" or "*REV-ERB*" or "*NR1D2*" or "*NR1D1*" or "*RORA*" or "*RORB*" or "*CSNK1E*" or "*NPAS2*" or "*TEF*" or "*TIMELESS*" or "*VIP*," "*VIPR2*." We then searched for related neuropsychiatric disorders as the outcome in conjunction with each of the genes listed above. This search resulted in 207 total manuscripts describing 204 genetic variants with association to the queried disorders (**Supplementary File 1**). We then grouped these genetic variants into four tiers based on the following criteria: *Tier 1*: significant association between the genetic variant and the above defined neuropsychiatric disorders across two or more previous reports, and minor allele frequency (MAF > 0.05) (n = 19 genetic variants); *Tier 2*: significant association between the genetic variant and the above defined neuropsychiatric disorders in at least one previous report, and MAF > 0.05 (n = 98 genetic variants); *Tier 3*: no significant association detected in previous report(s) (n = 24 genetic variants); *Tier 4*: MAF < 0.05 or no record of the MAF for African or Caucasian populations (n = 63 genetic variants). We then selected genetic variants from Tiers 1 and 2 for association analyses (n = 117 genetic variants). However, only a subset of these genetic variants were included on the genotyping array we had used to assess genetic variants in these cohorts. Thus, thirty-one genetic variants across nine circadian rhythm associated genes were included in primary analyses (**Supplementary File 1** and **Supplementary Table 2**).

Analyses

Genetic Association Analyses

Sociodemographic characteristics of the sample were summarized using standard descriptive statistics. Repeated measures mixed models were used to evaluate the association between each of the 31 genetic variants and PTSS outcomes over time and for the following potential interactions: sex × genetic variant, peritraumatic distress × genetic variant, and *FKBP5* (using the tagging allele, rs3800383) × genetic variant. [*FKBP5* was included in these analyses as a secondary measure for stress interactions, since *FKBP5* is a critical mediator of the hypothalamic pituitary axis and is highly associated with PTSS (46)]. Models were adjusted for potential confounding by age, education level, time since the traumatic event, and enrollment study site. Sex, distress, and *FKBP5*-dependent

effects were evaluated because of increasing evidence that such interactions are frequently present [e.g., (60–62)] and because such effects have been found for circadian rhythm associated genes previously (36–40). A dominant genetic model [two copies of the major allele (coded as 0) vs. one or more copies of the minor allele (coded as 1)] was used for all models. Marginal means corresponding to PTSS severity were derived from the fully adjusted models. False discovery rate (FDR) was controlled for in each analysis subset (e.g., main effects, stress interaction, sex interaction) separately. when determining statistical significance. All analyses were performed using SPSS and SAS software (v24; SPSS Inc. Chicago, IL; SAS 9.4, SAS Institute Inc., Cary, NC). Of note, the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

RNA Expression Analyses

RNA expression data was only available for the MVC cohort; thus analyses assessing the relationship between the genetic variant *TEF*rs5758324 and its effect on *TEF* mRNA expression were assessed in this cohort alone. The total cohort with RNA data ($n = 184$) was stratified by high and low distress and by rs5758324 major vs. minor allele. For each subgroup, bivariate analyses were used to assess the strength and direction of the relationship between *TEF* mRNA expression and PTSS severity. Due to non-normal distribution of *TEF* mRNA sequencing reads (Shapiro-Wilk test for normality, $p < 0.05$), Spearman's rank correlation coefficients were reported.

Bioinformatics Analyses

Linkage disequilibrium was assessed in this study using LDlink, a web-based application that enables exploration of population specific linkage structures based on data from 1000 Genomes Project Phase 3 and dbSNP build 142 (63).

RESULTS

Participants

Baseline characteristics of participants in the discovery and replication cohorts are shown in **Table 1**. The motor vehicle collision (MVC) cohort has been described previously (49). This study exclusively enrolled African American women and men ($n = 930$) who reported to the emergency department (ED) within 24 h of an MVC. The sexual assault (SA study) is an on-going study of multi-ethnic women who presented for emergency care within 72 h of SA. All participants enrolled in the SA study with PTSS outcome data available at the time of analyses were genotyped ($n = 274$). The major thermal burn injury (MThBI) study enrolled African American and European American women and men within 72 h of thermal burn injury ($n = 68$). Follow-up rates at the final 1 year follow-up timepoint were $\geq 85\%$ for all three studies.

Genetic Variants Selected for Primary Analyses

Because our cohort sizes were relatively small, to reduce the likelihood of type I and type II error we performed a candidate gene study using genetic variants in circadian rhythm associated genes previously associated with neuropsychiatric disorder vulnerability. These 31 genetic variants (**Table 2**), were identified via structured literature review (see Methods for details).

Relationship Between Circadian Rhythm Associated Genetic Variants and PTSS Severity Following Trauma Exposure

MVC Discovery Cohort Analyses

Repeated measures mixed models adjusting for ED study site, participant age, education level, and sex were used to assess whether any of the 31 variants identified in previous literature predicted PTSS severity after MVC. For each genetic variant, we assessed for main effect relationships with PTSS and for potential interactions with sex, peritraumatic distress, and *FKBP5* (using the rs3800373 tagging allele). Eight associations were identified (**Table 3**). Two of these, from the thyrotroph embryonic factor (*TEF*) gene, survived false discovery rate (FDR < 0.05) adjustment: *TEF*rs5758324*peritraumatic distress [$F_{(1,824)} = 10.54$, $p = 3.7 \times 10^{-2}$] and *TEF*rs738499**FKBP5* [$F_{(1,830)} = 10.05$, $p = 4.96 \times 10^{-2}$]. Stratified analyses demonstrating the direction and magnitude of effect for all associations in **Table 3** are presented in **Supplementary Figure 2**. Additionally, demographic data stratified by allele for *TEF*rs5758324 is presented in **Supplementary Table 3**.

Replication Analyses in SA and MThBI Cohorts

In repeated measures mixed model replication analyses, *TEF*rs5758324 significantly predicted PTSS severity in a peritraumatic distress-dependent manner in both the SA cohort [$F_{(1,257)} = 5.52$, $p = 0.020$, **Table 4**] and the MThBI cohort [$F_{(1,57)} = 5.72$, $p = 0.020$, **Table 4**]. Because the discovery cohort was comprised of only African American individuals (MAF = 0.35), we also assessed whether *TEF*rs5758324 predicted PTSS when limiting analyses to this strata alone (16.8% of SA cohort, 39.7% of MThBI cohort) (MAF range for different ancestry groups = 0.17–0.78; overall MAF = 0.48). We observed similar results for African American individuals as for the full cohort [SA, $F_{(1,33)} = 4.57$, $p = 0.040$; MThBI, $F_{(1,18)} = 7.52$, $p = 0.013$].

*TEF*rs5758324 Predicts PTSS Severity Following Trauma Exposure in a Stress Dependent Manner

Stratified analyses were performed to determine whether the direction and magnitude of the relationship between *TEF*rs5758324 and PTSS severity was similar in the three cohorts. Marginal means demonstrated that the direction of effect was similar across all three trauma exposures (**Figure 1**). Additionally, individuals with high distress and at least one copy of the *TEF*rs5758324 minor allele reported the highest PTSS severity compared to the other subgroups (**Figure 1**). This relationship persisted when adjusting for the time of trauma exposure, litigation status, and previous life trauma. Additionally, the effect of *TEF*rs5758324 and distress on PTSS severity following MVC did not seem to differ over the three posttraumatic timepoints measured ($F = 0.51$, $p = 0.598$). In the SA and MThBI cohorts, this difference was most

TABLE 2 | Genetic variants in circadian rhythm genes that have previously been shown to be associated with neuropsychiatric disorders and are the focus of primary analyses in the current study ($n = 31$ genetic variants).

Gene name	Gene symbol	Genomic location	SNP	Previous associations
Aryl hydrocarbon receptor nuclear translocator like	*ARNTL; BMAL1*	11p15	rs7107287	Anxious temperament (64); BD (65)
[-10pt]			rs1982350	Depression (66); BD (65)
			rs11022778	MDD, appetite changes (67); age at first suicide attempt (68)
			rs969485	Depression (66)
Clock circadian regulator	*CLOCK*	4q12	rs534654	Violent suicide attempts (68); BD (69)
			rs1801260	MDD in males (36); BD (70, 71); SAD (72); appetite disturbance (67); MDD (73); chronotype (74); evening activity (75, 76); insomnia during depression treatment (77); insomnia with MDD + BD (78)
Neuronal PAS domain protein 2	*NPAS2*	2q11.2	rs1562313	BD with seasonal pattern (79)
			rs12622050	BD with seasonal pattern (79)
			rs2305159	BD with seasonal pattern (79)
			rs6740935	MDD (73)
Period 2	*PER2*	2q37.3	rs6431590	MDD (17)
Period 3	*PER3*	1p36.23	rs10462018	MDD (73)
			rs228642	MDD (80)
RAR related orphan receptor A	*RORA*	15q22.2	rs4774388	Depression (66); BD (81)
			rs2414680	MDD (73)
			rs16943472	MDD (73)
			rs4775351	MDD (73)
			rs8023563	Depression (16)
			rs12906588	Depression (16)
			rs809736	Response to antidepressants (15)
			rs782931	BD (82)
			rs13329238	BD (81)
			rs9302215	BD (81)
			rs11071557	BD (81)
			rs12915776	BD (81)
			rs8041466	BD (81)
			rs34720147	BD (81)
RAR related orphan receptor B	*RORB*	9q22	rs7022435	BD (83)
TEF, PAR bZIP transcription factor	*TEF*	22q13.2	rs738499	Depression (74, 84–87); sleep disturbances (88)
			rs5758324	MDD (73)
Timeless circadian clock	*TIMELESS*	12q13.3	rs11171856	violent suicide attempts (68)

MDD, major depressive disorder; BD, bipolar disorder.

pronounced among African American individuals. In sum, these data support the hypothesis that, *TEF*rs5758324 has a stress-dependent influence on PTSS severity, across a range of trauma exposures and ethnicities.

*TEF*rs5758324 Shows the Strongest Association With PTSS Compared to Other Genetic Variants in the Surrounding Region

To determine whether the observed association for *TEF*rs5758324 is likely attributable to the *TEF* gene locus (vs. another gene in linkage disequilibrium with *TEF*rs5758324), we assessed for an association between PTSS and available genetic variants (i.e., variants in our genotyping array) within a 250 kb span surrounding *TEF*rs5758324. The strongest stress-dependent association with PTSS severity originated from three of the four genetic variants mapping to the *TEF* gene (rs5751086, $p = 3.8 \times 10^{-3}$; rs738499, $p = 3.3 \times 10^{-3}$; rs5758324, $p = 1.2 \times 10^{-3}$; **Figure 2**). These genetic variants are all located in intronic or upstream regions of *TEF* (**Figure 2**) and are in high linkage disequilibrium with each other (**Supplementary Figure 3**).

*TEF*rs5758324 Affects the Relationship Between *TEF* mRNA Expression and PTSS Severity

To evaluate potential functional effects of *TEF*rs5758324, we used RNA sequencing data available in a subset of individuals ($n = 184$) in the MVC cohort to assess the relationship between *TEF* mRNA expression levels and PTSS severity. To perform this

TABLE 3 | Relationship between genetic variants in circadian rhythm genes and posttraumatic stress symptom severity following motor vehicle collision trauma in African American individuals ($n = 930$).

Gene name	SNP	Alleles	Interaction	F statistic	*p*-value*	*p*-value# (FDR adj.)
RORB	rs7022435	G/A	-	5.22	$2.26 * 10^{-2}$	0.315
BMAL1	rs969485	A/G	-	5.13	$2.38 * 10^{-2}$	0.315
RORA	rs4774388	T/C	-	4.70	$3.05 * 10^{-2}$	0.315
RORA	rs4774388	T/C	sex	6.18	$1.31 * 10^{-2}$	0.406
NPAS2	rs12622050	G/A	distress	5.10	$2.41 * 10^{-2}$	0.250
TEF	rs5758324	T/G	distress	10.54	$1.20 * 10^{-3}$	**$3.70 * 10^{-2}$**
TEF	rs738499	T/G	distress	8.69	$3.30 * 10^{-3}$	$5.10*10^{-2}$
TEF	rs738499	T/G	*FKBP5*	10.05	$1.60 * 10^{-3}$	**$4.96 * 10^{-2}$**

Relationships examined include genetic variant main effects, and interactions between the genetic variant and participant sex, peritraumatic distress, or FKBP5 tagging allele, rs3800373.
** p-value generated via repeated measures mixed models (6 week, 6 months, 1 year), adjusted for age, sex, emergency department enrollment site, education, and time following motor vehicle collision. A dominant genetic model was used for all genetic polymorphisms. # False discovery rate (FDR) adjusted p-values.*
Bold values indicate significance at the $p < 0.05$ threshold.

TABLE 4 | Relationship between genetic variants in circadian rhythm genes and posttraumatic stress symptoms in individuals following sexual assault (SA, $n = 274$) and major thermal burn injury (MThBI, $n = 68$) trauma.

			SA cohort		MThBI cohort	
Gene name	**SNP**	**Interaction**	***p*-value (All)***	***p*-value (AA)***	***p*-value (All)***	***p*-value (AA)***
RORB	rs7022435	–	0.641	0.112	*NA*	*NA*
BMAL1	rs969485	–	0.663	0.546	0.351	0.443
RORA	rs4774388	–	0.579	0.630	0.601	0.928
RORA	rs4774388	sex	–	–	0.505	0.899
NPAS2	rs12622050	distress	0.171	0.131	0.296	0.333
TEF	rs5758324	distress	**0.020**	**0.040**	**0.020**	**0.013**
TEF	rs738499	distress	0.233	0.421	0.524	0.206
TEF	rs738499	*FKBP5*	0.995	0.665	0.485	0.909

Only genetic variants shown to be associated with posttraumatic stress symptoms following motor vehicle collision (MVC) were assessed in these cohorts.
**p-value generated via repeated measures mixed models (6 week, 6 months, 1 year), adjusted for age, sex, emergency department enrollment site, education, and time following sexual assault (SA) or major thermal burn injury (MThBI). AA, African American individuals only; NA, genetic variant not available.*
Bold values indicate significance at the $p < 0.05$ threshold.

analysis, we first stratified individuals with RNA data based on their peritraumatic distress level and the presence or absence of at least one copy of the *TEF*rs5758324 minor allele. In distressed individuals with at least one copy of the minor allele, we observed a statistically significant positive correlation between *TEF* mRNA and PTSS severity (**Table 5**, **Figure 3**). In the other three strata, there were no statistically significant correlations between *TEF* mRNA and PTSS severity (**Table 5**). These data indicate that the *TEF*rs5758324 minor allele strengthens the relationship between *TEF* RNA expression and subsequent PTSS severity in stressed individuals.

DISCUSSION

In the present study, we searched among genetic variants in circadian pathway genes associated with neuropsychiatric disorders for variants that influence posttraumatic stress symptom severity after traumatic stress. We identified a genetic variant in the *TEF* gene, rs5758324, that had a stress-dependent influence on posttraumatic stress symptom severity across three different trauma exposures (MVC, SA, and MThBI) and across multiple ethnicities, such that individuals with peritraumatic distress and at least one copy of the minor allele had the highest levels of PTSS over time. Additional *TEF* variants in LD with *TEF*rs5758324 were also significantly associated with PTSS in a stress-dependent manner, suggesting that *TEF*rs5758324 might be an important risk locus for PTSS. Further, a strong positive relationship between *TEF* mRNA expression and PTSS severity was observed in individuals with high levels of peritraumatic distress and at least one copy of the *TEF*rs5758324 minor allele, suggesting that the variant may be functional.

TEF is one of several key regulatory transcription factors within the circadian rhythm pathway. *TEF*, along with several other key circadian-associated transcriptional activators, binds at D-box promoter sites to promote transcription of a number of clock-controlled genes as well as key genes in the core circadian feedback loop including *NR1D1*, *NR1D2*, *PER*, and *CRY* (89–91). How *TEF* polymorphisms might influence the pathogenesis of PTSS remains poorly understood. Such polymorphisms may affect sleep quality in the days or weeks after trauma, contributing to PTSS onset or preventing remittance via a variety of mechanisms (92, 93). In addition, *TEF* has been found to

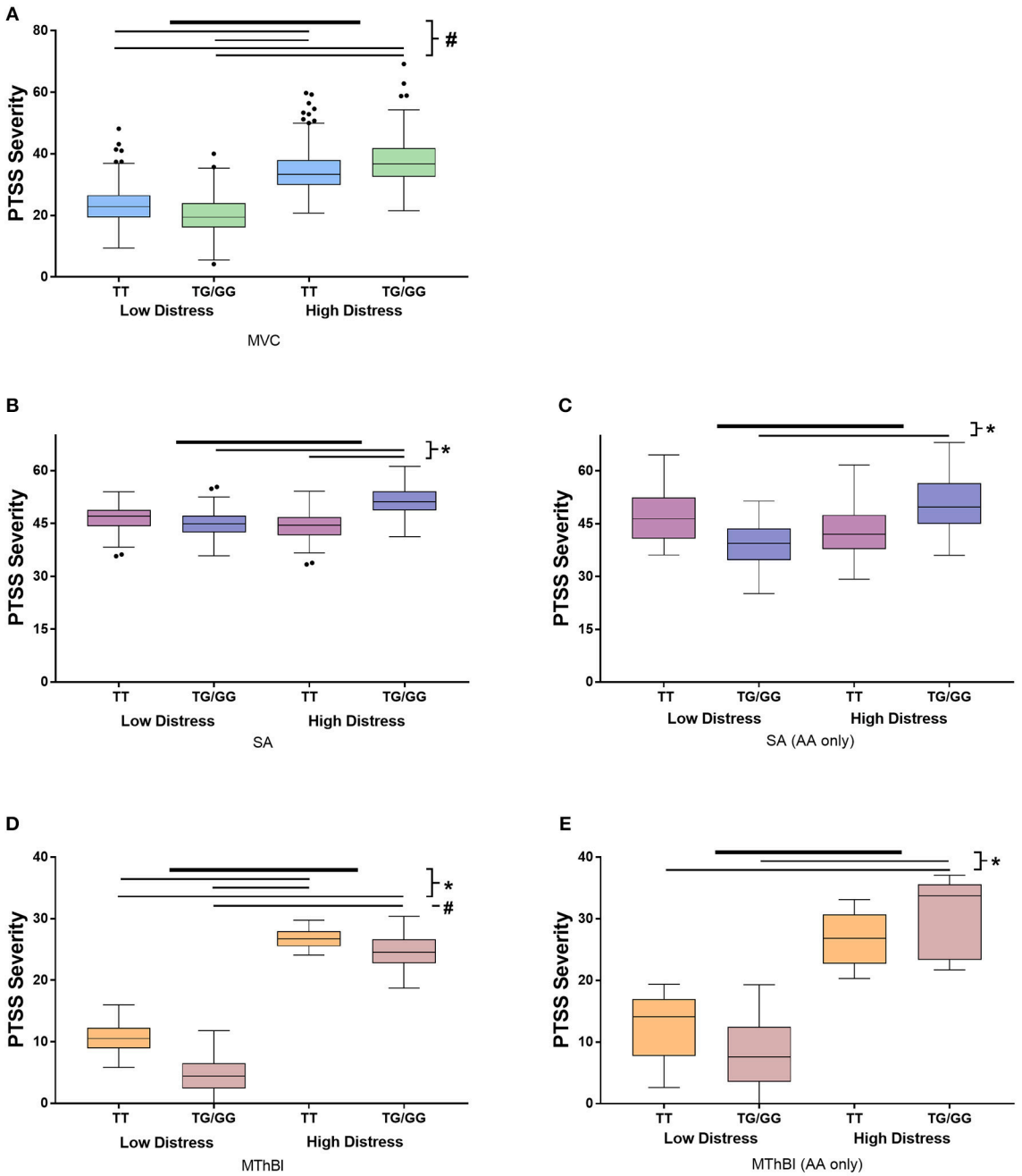

FIGURE 1 | Results of stratified analyses assessing the influence of the interaction between *TEF*rs5758324 and peritraumatic distress on posttraumatic stress symptom (PTSS) severity following motor vehicle collision (MVC), sexual assault (SA) and major thermal burn injury (MThBI) trauma exposures. PTSS severity in individuals with low and high distress and the major or at least one copy of the minor allele at *TEF*rs5758324 shown in all panels. Specific cohort data shown as follows: **(A)** MVC study participants ($n = 931$). All participants in this study were African American. **(B)** SA study participants ($n = 274$). **(C)** African American individuals from the SA study ($n = 46$). **(D)** MThBI study participants ($n = 68$). **(E)** African American individuals from the MThBI study ($n = 27$). Outliers defined using Tukey criteria are represented by black dots. $^{*}p$-value < 0.05, $^{\#}p$-value < 0.001.

influence brain levels of serotonin and dopamine through the clock-controlled gene, *PDXK* (94). Future studies defining the role of *TEF* in PTSS pathogenesis, such as knock-out or gene silencing animal studies, or the evaluation of peritraumatic sleep patterns among distressed individuals with and without the *TEF*rs5758324 minor allele is warranted.

While previous studies have identified a relationship between polymorphisms in the *TEF* gene and PTSS-associated neuropsychiatric disorders, most focused on *TEF*rs738499, a polymorphism in high LD with *TEF*rs5758324 (D' = 0.987, $R^2 = 0.272$). (This allele was associated with PTSS development in our discovery cohort, but did not replicate in SA and MThBI

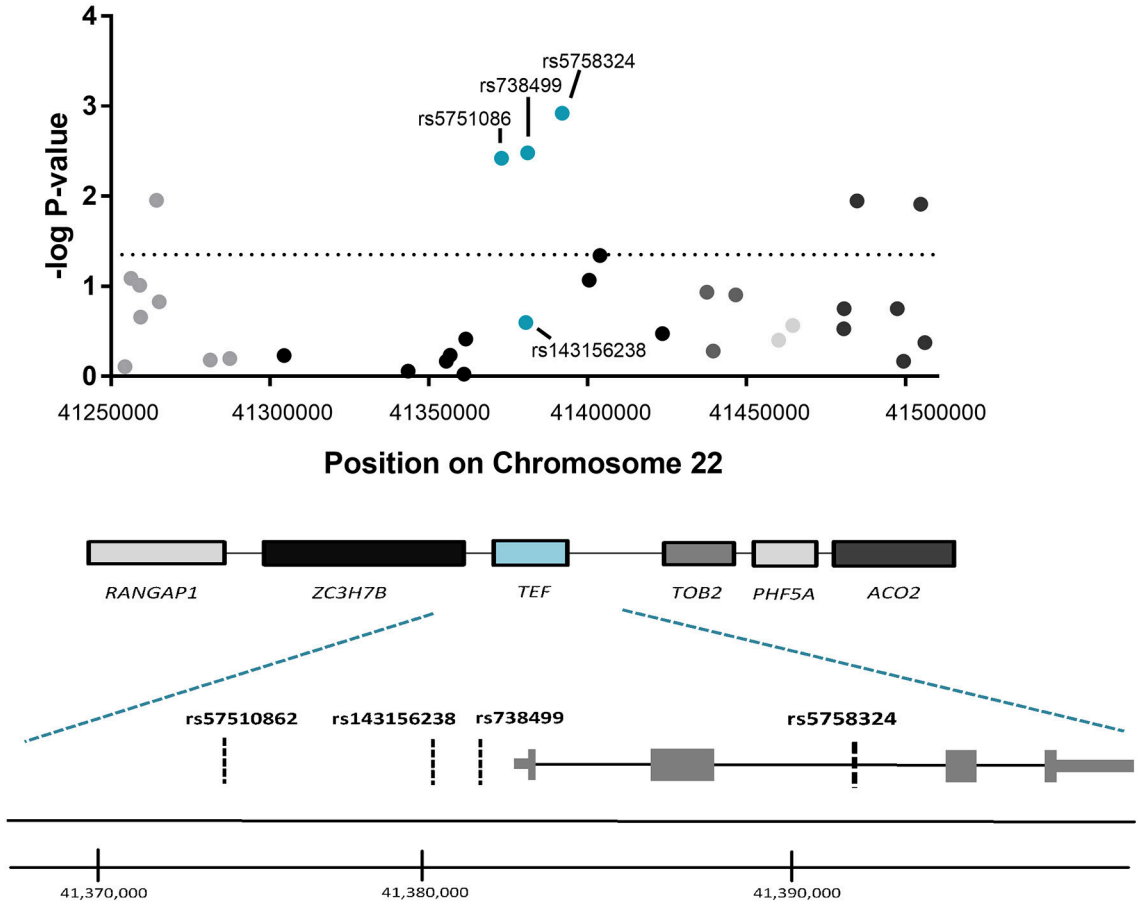

FIGURE 2 | Association between genetic variants in the genomic region surrounding *TEF*rs5758324 and PTSS severity following motor vehicle collision (MVC) trauma. **(Top)** The –log *p*-values of the peritraumatic distress -dependent association between each genetic variant (represented by a dot on the graph) and PTSS severity following MVC were plotted vs. the location of each genetic variant on Chromosome 22. The horizontal dotted line indicates a significance threshold of $p = 0.05$. Corresponding genes mapping to the genomic region are indicated below the graph and shading/colors are coordinated between the dots representing genetic variants and the gene to which they map. **(Bottom)** Magnified schematic of the *TEF* gene indicating both isoforms for the *TEF* transcript, the relative location of exons and introns, and the relative location of each genetic variant assessed within the *TEF* gene. (*TEF* isoform 1: ENST00000266304.8 and *TEF* isoform 2: ENST00000406644.7). Genome coordinates refer to GRCh38/hg38 Assembly.

TABLE 5 | Effect of stress and *TEF*rs5758324 on the correlation between *TEF* mRNA levels and PTSS severity following motor vehicle collision (MVC, $n = 184$).

Distress level	Allele	Correlation between *TEF* mRNA and PTSS severity following MVC	
		Correlation coefficient[a]	*p*-value
Low distress	TT	−0.005	0.956
	TG/GG	−0.070	0.441
High distress	TT	0.048	0.599
	TG/GG	**0.183**	**0.035**

[a] *Spearman's rho.*
Bold values indicate significance at the p < 0.05 threshold.

survivors). Previous literature has identified an association between *TEF*rs738499 and depression (74, 84–87) and sleep disturbances (88). Interestingly, an expanded study of the initial cohort that identified an association between *TEF*rs738499 and depression failed to replicate the relationship (73), suggesting that *TEF*rs738499 might not be a functional allele or might not have relevance to all populations or types of depression. The same expanded study that failed to replicate the association for *TEF*rs738499 identified an association at the trend-level for *TEF*rs5758324. Consistent with the hypothesis that *TEF*rs5758324 is functional, publicly available data from the GTEx consortium (95) indicates that in 44 cell lines/tissues examined, *TEF*rs5758324 is an expression quantitative trait locus (eQTL). Additionally, using data cataloged by Metamoodics (96), *TEF* mRNA is increased in the frontal cortex of individuals with neuropsychiatric disorders (though this online database did not distinguish genetic variant effects on mRNA expression).

We did not detect a main effect relationship between *TEF* alleles and PTSS outcomes similarly to previous studies. Instead, we detected a gene × environment interaction, where stressed individuals with at least one copy of the minor allele reported the highest PTSS severity. Such gene × stress

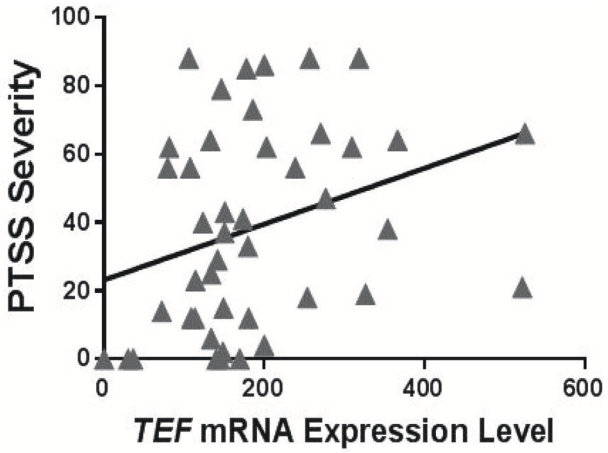

FIGURE 3 | Relationship between circulating *TEF* mRNA expression levels and PTSS severity following motor vehicle collision (MVC) trauma in individuals who reported high peritraumatic distress in the early aftermath of trauma exposure and who had at least one copy of the *TEF*rs5758324 minor allele. Shown is representative data from PTSS levels measured 6 months following MVC ($n = 50$; Spearman's rho $= 0.386$, $p = 0.009$). No relationship between *TEF* mRNA and PTSS severity was observed for other subgroups of individuals: high peritraumatic distress and *TEF*rs5758324 major allele ($n = 45$; Spearman's rho $= -0.007$, $p = 0.967$), low peritraumatic distress and *TEF*rs5758324 minor allele ($n = 42$; Spearman's rho $= -0.074$, $p = 0.642$), low peritraumatic distress and *TEF*rs5758324 major allele ($n = 38$; Spearman's rho $= -0.087$, $p = 0.604$).

interactions are being increasingly identified (97–99). For instance, the relationship between *FKBP5* alleles and PTSS/PTSS related neuropsychiatric outcomes have consistently been shown to be stress dependent (46, 47, 100–102). Future studies should consider such interactions when examining genetic associations between *TEF* or other circadian rhythm genes and neuropsychiatric disorders.

A number of limitations should be considered when interpreting this manuscript. First, this study used a candidate gene approach to test a specific hypothesis regarding the role of circadian rhythm genetic variants in predicting PTSS development. Many in the mental health field are moving away from candidate gene studies, in favor of GWAS approaches. However, given their ability to evaluate pathways with high pre-test probability, test for interactions, replicate results across multiple studies and ethnicities, and use multilayered data analyses to evaluate for evidence of functionality, we believe that they still have merit and utility to advance the field. Second, our functional assessment of *TEF*rs5758324 using mRNA expression analyses was limited to blood expression levels. *TEF* is widely expressed throughout the body, with highest expression in the brain. Therefore, it is possible that blood measurements of expression are not an accurate proxy for brain expression. A previous report showed that the transcriptome of some central nervous tissues and the blood overlap (103), suggesting that *TEF* blood expression might approximate nervous tissue expression. However, further studies are needed to directly address this possibility. Third, we only assessed association between four *TEF* variants and PTSS development. The use of targeted sequencing methods could provide further granulation of genetic variation across the *TEF* gene and enable us to pinpoint the most influential genetic variants in this genomic region (in addition to *TEF*rs5758324). Fourth, we did not examine whether additional factors related to stress or the circadian clock, such as stress hormone levels and/or sleep quality mediates the relationship between *TEF*rs5758324 and PTSS outcomes. However, such analyses would be of interest for future studies. Fifth, the list of candidate SNPs identified via literature search, that were analyzed for association with PTSS in the current study, was limited in scope due to the reliance on previous studies showing an association between a circadian rhythm associated gene and a PTSS related disorder. This study design resulted in an under sampling of SNPs in key circadian rhythm genes such as *CRY1* and *PER1*. In the future, higher-powered studies would benefit from assessing tagging SNPs across all circadian rhythm associated genes to identify additional circadian rhythm SNPs that might predict PTSS following trauma exposure. Sixth, we do not know from which blood cell component our RNA expression originated, thus limiting our ability to make inferences about the origin of *TEF* mRNA in this study. Seventh, only approximately one-third of African American individuals carry the *TEF*rs5758324 minor allele. In combination with African Americans comprising only a subset of the SA and MThBI cohorts, stratified analyses were likely underpowered. Finally, the genetic variant in *RORA*, rs8042149, which was identified in the first GWAS for PTSD (8) was unfortunately not included on our genotyping array, thus we were not able to assess whether this particular allele predicted PTSS in our trauma cohorts. Additionally, there was very little LD between our *RORA* variants and *RORA*rs8042149. Therefore, this study should not be considered a failed replication.

In conclusion, the above data, from multiple trauma exposures and across ethnicities, suggest that individuals with the *TEF*rs5758324 minor allele and high levels of peritraumatic distress experience more severe PTSS than individuals with the *TEF*rs5758324 major allele. Further, the above data provide preliminary evidence that *TEF*rs5758324 is functional. Further studies are needed to elucidate potential mechanisms mediating these relationships, as improved understanding of such mechanisms could contribute to improved PTSS prevention and treatment.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board at each participating hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board at the data coordinating center and at each enrolling hospital.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

SL and CZ: conceived the manuscript; SL, YP, MM, CZ, LJ, CR, JB, MC, and AT: performed analyses and made figures/tables; SL and SM: contributed to manuscript design and writing; JS, MK, PH, CL, TD, ED, KB, ML, JWS, and BC: assisted with study design and were responsible for data collection at individual ED sites.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00597/full#supplementary-material

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Inflammatory Measures in Depressed Patients With and Without a History of Adverse Childhood Experiences

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Background: Major depressive disorder (MDD) is a complex psychiatric condition with different subtypes and etiologies. Exposure to adverse childhood experiences (ACE) is an important risk factor for the development of MDD later in life. Evidence suggests that pro-inflammatory processes may convey this risk as both MDD and ACE have been related to increased levels of inflammation. In the present study, we aimed to disentangle the effects of MDD and ACE on inflammation levels.

Methods: Markers of inflammation (plasma interleukin(IL)-6 and high sensitive C-reactive protein (hsCRP) concentrations, white blood cell (WBC) count and a composite inflammation score (CIS) combining all three) were assessed in 23 MDD patients with ACE, 23 MDD patients without ACE, 21 healthy participants with ACE, and 21 healthy participants without ACE (mean age: 35 ± 11 (SD) years). None of the patients and participants was taking psychotropic medication. ACE was assessed with the Early Trauma Inventory (ETI) and was defined as moderate to severe exposure to sexual or physical abuse.

Results: Group differences in the different inflammatory measures were observed. MDD patients with ACE showed significantly higher IL-6 concentrations ($p = 0.018$), higher WBC counts ($p = 0.003$) and increased general inflammation levels as indicated by the CIS ($p = 0.003$) compared to healthy controls. In contrast, MDD patients without ACE displayed similar inflammation levels to the control group ($p = 0.93$).

Conclusion: We observed elevated inflammation in MDD patients with a history of ACE, which could indicate a subtype of "inflammatory depression". Accordingly, MDD patients with ACE might potentially benefit from anti-inflammatory therapies.

Keywords: acute-phase protein, childhood adversity, childhood maltreatment, depression, inflammation, pro-inflammatory cytokine

INTRODUCTION

Major depressive disorder (MDD) is a frequent and heterogenic disorder and despite numerous studies performed over the last decades, there are still inconsistencies in clinical findings regarding the pathological mechanisms contributing to the development of MDD (1). Therefore, greater understanding of the biological processes and pathways underlying the pathophysiology of depression is of key importance for the development of early interventions and personalized therapies.

Adverse childhood experiences (ACE) have been shown to predispose to the development of MDD later in life (2, 3) and in addition induce greater risk for acquiring several somatic conditions, including cardiovascular disease (CVD) (3). A large body of evidence suggests that ACE is linked to a chronic pro-inflammatory state in adulthood (4–6). Alterations in the dynamics of the neuroendocrine stress response likely contribute to the manifestation of a pro-inflammatory immune phenotype in these individuals (7). It has been suggested that stressors occurring early in life can be biologically embedded through epigenetic modifications in stress-related genes (8) and program the immune system to become hyper-responsive in response to challenge with diminished sensitivity to the inhibitory effect of glucocorticoids (9).

Chronic inflammation is characterized by elevated levels of pro-inflammatory cytokines, acute phase proteins, and increases in white blood cell (WBC) numbers (10–12). Several studies reported higher circulating levels of inflammatory mediators, such as the pro-inflammatory cytokine IL-6 and the acute phase-protein C-reactive protein (CRP) (4, 5), and increased WBC counts (13, 14) in adults exposed to early adversity. Because chronic inflammation is associated with both ACE and several physical and psychiatric conditions, including MDD (15–18), it has been proposed as a key mechanism through which severe stress exposure during childhood can influence health outcomes throughout the lifespan (6, 19). This notion is further supported by the observation that increased activation of pro-inflammatory pathways (reflected by increased circulating levels of CRP and IL-6) precedes the development of depressive symptoms (17), and by studies that report that inflammation is more pronounced in a subgroup of MDD patients that are exposed to ACE (20–23).

The overall goal of the present study was to replicate previous findings regarding associations between ACE, MDD and inflammation, and to further disentangle the effects of MDD and ACE on inflammation using a well-controlled, full factorial design including four carefully diagnosed groups of healthy participants and MDD patients with and without a history of ACE. None of the patients and participants was taking psychotropic medication. We hypothesized accumulative effects of MDD and ACE on inflammation levels.

MATERIAL AND METHODS

Participants

Patients and healthy participants were recruited by public postings and from our specialized affective disorder unit at the Department of Psychiatry and Psychotherapy, Campus Benjamin Franklin, Charité -Universitätsmedizin Berlin. All participants provided written informed consent. Healthy participants and outpatients received monetary compensation for their participation. The study was approved by the local ethical committee.

Depressed patients were included if they fulfilled criteria for MDD as assessed with the Structured Clinical Interview for DSM-IV axis I (SCID-I) (24) to validate psychiatric diagnoses. In addition, current depressive symptoms were captured by the Montgomery Asberg Depression Rating Scale (MADRS) (25, 26) and the Beck Depression Inventory (BDI) (27).

ACE was assessed by using a semi-structured interview, the Early Trauma Inventory (ETI) (28, 29), and was defined as repeated physical or sexual abuse at least once a month over one year or more before the age of 18.

In the MDD groups, schizophrenia, schizoaffective disorder, bipolar disorder, depressive disorder with psychotic features, dementia, eating disorders, panic disorder, alcohol or drug dependence led to exclusion. Healthy participants with and without ACE were free of any current mental disorder. Further exclusion criteria for all participants were CNS relevant diseases, neurological diseases, severe somatic diseases, diabetes type 1 and 2, steroid diseases, hypertonia, current infections, pregnancy and the intake of psychotropic medication. Physical health criteria were checked by physical examination, clinical interview and a complete blood count (CBC).

The study sample comprised 23 MDD patients with ACE (MDD+/ACE+), 23 MDD patients without ACE (MDD+/ACE–), 21 participants with ACE but no current, or lifetime MDD (MDD–/ACE+) and 21 participants with no current or lifetime MDD and no childhood adversity (MDD–/ACE–, healthy comparison group).

Study Protocol

All patients and participants underwent one study visit including psychiatric and medical diagnostic by physical examination, blood sampling and clinical interviews including SCID-I and MADRS as well as assessment of ACE using the ETI. Afterwards they completed a MDD related questionnaire (BDI). Blood samples were sent immediately to the laboratory of the Institute of Medical Psychology, Campus Mitte, Charité–Universitätsmedizin Berlin, Germany, and to the Labor Berlin–Charité Vivantes GmbH, Berlin, Germany, for further analyses.

Inflammatory Measures

Plasma IL-6 concentrations were analyzed using a commercially available high sensitivity ELISA kit (eBioscience), according to the manufacturer's instructions. The limit of detection was 0.007 pg/ml. The intra- and inter-assay coefficients of variability for plasma IL-6 measurements were 10 and 12%, respectively. Plasma hsCRP concentrations were analyzed using a commercially available Instant ELISA kit (eBioscience), according to the manufacturer's instructions. The limit of detection was 3 pg/ml. The intra- and inter-assay coefficients of variability for plasma hsCRP measurements were 6 and 8%, respectively. WBC counts were obtained from a standard clinical complete blood count panel using a Sysmex XN 1000 (Sysmex).

Statistics

General linear models and Chi^2 tests were used to compare groups concerning demographics and clinical data (see **Table 1**). *Post hoc* tests (Bonferroni) were conducted when applicable. IL-6 and hsCRP measures were first log transformed to normalize distributions. Since IL-6 and hsCRP concentrations and WBC count are established measures of pro-inflammatory activity, represent three biologically related components of inflammation [i.e., (a) pro-inflammatory cytokines (b) acute phase proteins and (c) increased WBC numbers], and inter-correlated with each other (all r's >0.2), we combined these measures into a single composite measure. Principal-component analysis identified one single factor, the composite inflammation score (CIS), accounting for 48% of the variance in analyte determinations. A common factor takes full advantage of the predictive values of the three measures, while minimizing measurement errors of the single components (21).

General linear models were used to compare groups regarding inflammatory measures. In order to investigate the groups effects on inflammatory measures in more detail, and because we expected the lowest inflammation levels in the control group, we studied a priori defined contrasts between the controls and the three study groups.

Many potential cofounders were excluded by design (see above). However, additional adjusted analysis included covariates that differed significantly between the four groups (i.e., BMI and smoking see **Table 1**).

Data analysis was performed using the SPSS statistical software (SPSS 23.0, Inc., Chicago, IL, USA). The significance level was set at $p < 0.05$ for all applied analysis.

Missing Data

Complete blood counts were missing for 8 individuals (4 MDD+/ACE+, 2 MDD+/ACE–, 1 MDD–/ACE+ and 1 MDD–/ACE–). For IL-6, a measurement was missing for 1 MDD patient without ACE (MDD+/ACE–). General linear models indicated there were no group differences regarding the number of missing biological measurements ($p = 0.42$).

RESULTS

Sample Characteristics

Table 1 summarizes group demographics and clinical characteristics. In accordance with our recruitment, MDD patients and healthy participant with ACE (MDD+/ACE+, MDD–/ACE+) had significantly higher total ETI scores compared to the MDD patients without ACE (MDD+/ACE–) and the control group (MDD–/ACE–). MDD patients with and without ACE did not differ in depression severity and both groups had higher depression scores compared to healthy individuals with and without ACE. MDD patients with ACE had a higher BMI compared to MDD patients without ACE and smoked more than healthy controls. No group differences were observed in age, sex and educational level.

Inflammatory Measures

As presented in **Table 2**, we identified significant group effects on IL-6 [$F_{(3,83)} = 3.32$, $p = 0.024$, $\eta^2 = 0.11$], CRP concentration [$F_{(3,84)} = 3.10$, $p = 0.031$, $\eta^2 = 0.10$] and WBC count [$F_{(3,76)} = 3.44$, $p = 0.021$, $\eta^2 = 0.12$]. To investigate these effects in more detail, we studied a priori defined contrasts between controls and the different study groups (see **Table 3**). We observed significantly higher IL-6 concentrations ($p = 0.018$) and WBC counts ($p = 0.003$) in MDD patients with ACE compared to healthy controls, also after controlling for BMI and smoking (IL-6, $p = 0.044$; WBC count, $p = 0.048$). CRP levels were significantly higher in healthy individuals with ACE ($p = 0.031$). However, this effect was no longer significant after controlling for BMI and smoking ($p = 0.052$). Untransformed and unadjusted mean group values for IL-6, hsCRP and WBC counts are presented in **Figures 1A–C**.

There was a significant group effect on the CIS [$F_{(3,75)} = 4.76$, $p = 0.004$, $\eta^2 = 0.16$, **Table 2**]. Contrasts between the controls and the different study groups showed that inflammation levels tended to be increased in healthy participants with ACE ($p = 0.073$, **Table 3**) and were significantly higher in MDD patients with ACE compared to healthy controls ($p = 0.003$, **Table 3**), also after controlling for BMI and smoking ($p = 0.034$, **Table 3**). Unadjusted mean group CIS values are depicted in **Figure 1D**.

DISCUSSION

With the present study we aimed to disentangle the effects of MDD and ACE on alterations in levels of inflammation by using well-controlled, defined and discrete groups of adults with and without a history of ACE and an MDD diagnosis. Confirming our hypothesis, we observed the highest inflammation levels in MDD patients with a history of ACE. These results replicate prior research showing that inflammation is elevated in a subgroup of MDD patients exposed to ACE (21). Our results are also in line with previous studies reporting that elevations in inflammatory measures observed in MDD patients are associated with childhood trauma (22, 23, 30). Another study comparing cytokine levels between healthy controls and MDD patients with and without a history of ACE found the highest levels of 13 different cytokines in the ACE exposed MDD patients (31). However, in contrast to our findings, no increases in plasma levels of IL-6 were observed in this subgroup of MDD patients. A possible explanation for this discrepancy could be the use of different assay methodology.

Group differences were not completely homogenous regarding the different inflammatory measures that we assessed. While IL-6 concentrations and WBC counts were elevated in MDD patients with ACE, higher CRP concentrations were seen in healthy individuals exposed to ACE compared to healthy controls. This last finding is supported by a recent meta-analysis suggesting that the association between childhood trauma and inflammatory measures, including CRP, is not moderated by the presence of a psychiatric diagnosis (5). However, our finding that CRP is increased in healthy individuals exposed to ACE

TABLE 1 | Demographic and clinical characteristics of healthy participants and depressed patients without ACE (MDD–/ACE–, MDD+/ACE–) and healthy participants and depressed patients with ACE (MDD–/ACE+, MDD+/ACE+).

	MDD–/ACE– ($n = 21$)	MDD+/ACE– ($n = 23$)	MDD–/ACE+ ($n = 21$)	MDD+/ACE+ ($n = 23$)	Statistics (GLM, Chi^2)
Age (SD)	33.90 (9.77)	32.61 (11.74)	34.05 (10.53)	38.09 (11.36)	$p = 0.36$
Sex (m/f)	8/13	5/18	6/15	9/14	$p = 0.55$
BMI (SD)	23.23 (3.56)[a,b]	21.49 (2.87)[a]	23.79 (3.14)[a,b]	25.05 (2.93)[b]	$p = 0.003$
Smoking (y/n)	4/17 [a]	5/18 [a]	7/14 [a,b]	14/9 [b]	$p = 0.012$
Educational level (%)					
Lower/Intermediate	23.8%	21.7%	28.6%	47.8%	$p = 0.21$
Upper Secondary School	76.2%	78.3%	71.4%	52.2%	
DEPRESSIVE SYMPTOMS					
BDI (SD)	1.05 (1.56)[a]	25.41 (8.96)[b]	4.64 (4.75)[a]	26.81 (8.62)[b]	$p < 0.001$
MADRS score (SD)	0.62 (0.81)[a]	28.26 (5.71)[b]	1.67 (1.83) [a]	27.41 (8.01)[b]	$p < 0.001$
ADVERSE CHILDHOOD EXPERIENCES					
ETI sum score (SD)	12.67 (21.13) [a]	195.39 (205.48)[a]	619.62 (371.74)[b]	752.09 (482.57)[b]	$p < 0.001$

[a,b]Groups with values that do not share a superscript within the same line of text are significantly different from each other. ACE, Adverse childhood experiences; BMI, Body mass index; BDI, Becks Depression Index; ETI, Early Trauma Interview; GLM, General linear model; MADRS, Montgomery Asberg Depression Rating Scale; MDD, Major depressive disorder; SD, Standard deviation.

TABLE 2 | Mean values of the inflammatory measures of healthy participants and depressed patients without ACE (MDD–/ACE–, MDD+/ACE–) and healthy participants and depressed patients with ACE (MDD–/ACE+, MDD+/ACE+).

	MDD–/ACE–	MDD+/ACE–	MDD–/ACE+	MDD+/ACE+	GLM
IL-6 (Ln) pg/ml (SE)	−1.28 (0.25)	−1.44 (0.30)	−1.12 (0.29)	−0.43 (0.14)	$F_{(3,83)} = 3.32$ $p = 0.024$
hsCRP (Ln) mg/L (SE)	−1.96 (0.22)	−2.12 (0.21)	−1.20 (0.29)	−1.49 (0.23)	$F_{(3,84)} = 3.10$ $p = 0.031$
WBC count 10^9/L (SE)	6.01 (0.27)	6.52 (0.53)	6.97 (0.53)	8.09 (0.50)	$F_{(3,76)} = 3.44$ $p = 0.021$
CIS (SE)	−0.36 (0.16)	−0.39 (0.23)	0.18 (0.24)	0.57 (0.20)	$F_{(3,75)} = 4.76$ $p = 0.004$

ACE, Adverse childhood experiences; Composite inflammation score; GLM, General linear model, MDD, Major depressive disorder; SE, Standard error; WBC, White blood cell.

was no longer significant after adjusting for BMI and smoking. As previously reported by others, unhealthy lifestyle factors like increased BMI and smoking are associated with ACE (32) and have been shown to have an effect on inflammation (33, 34). Therefore, these lifestyle factors could have contributed to the observed elevations in CRP concentrations.

Altogether, the data presented here support the hypothesis that ACE might be a risk factor for developing a MDD later in life, and that this risk is partly mediated by increases in activation of pro-inflammatory pathways (19). In line with this, inflammation levels did not differ between MDD patients without ACE and healthy controls, suggesting that biological mechanisms, other than inflammation, might play a more prominent role in the pathogenesis of depression in these patients. Although previous research has shown increased inflammatory measures in depression (15, 17, 18), in most studies the effects of ACE have not been taken into account.

ACE is not only a risk factor for the development of depression. Also other psychiatric disorders, such as post-traumatic stress-disorder (PTSD), anxiety disorders, and bipolar disorder have been associated with a history of ACE (35, 36) and are as well related to increased inflammation (37, 38). However, until now, only few studies systematically investigated the separate and interactive effects of disease status and a history of ACE on inflammation (39–41). Therefore, future research should attempt to further identify the role of ACE in activating inflammatory pathways in these psychiatric conditions.

Findings from this study are limited by the use of a cross sectional design and the relatively modest sample size, which might have led to insufficient power. Also our study sample included relatively more female than male participants, indicating that our results might be impacted by gender bias. However, the groups did not differ significantly regarding the female to male ratio. In addition, the number of immune parameters that we assessed was limited, and future studies should include additional measures of acute-phase proteins, cytokines, and immune cell characteristics in order to gain better understanding of the biological processes and pathways underlying the inflammation-related pathophysiology of depression in patients with and without a history of ACE.

TABLE 3 | Mean differences in inflammatory measures between each of the three study groups (MDD+/ACE–, MDD–/ACE+ and MDD+/ACE+) and control group (MDD–/ACE–) before (unadjusted) and after adjusting for BMI and smoking.

	MDD–/ACE–	MDD+/ACE–	MDD–/ACE+	MDD+/ACE+
IL-6 (Ln) pg/ml				
Unadjusted (95% CI)	–	−0.16 (−0.87, 0.54) $p = 0.65$	0.16 (−0.55, 0.87) $p = 0.65$	0.85 (0.15, 1.54)* $p = 0.018$
BMI & Smoking (95% CI)	–	−0.10 (−0.83, 0.63) $p = 0.79$	0.14 (−0.58, 0.86) $p = 0.70$	0.77 (0.02,1.53)* $p = 0.044$
hsCRP (Ln) mg/L				
Unadjusted (95% CI)	–	−0.16 (−0.83, 0.51) $p = 0.64$	0.76 (0.07, 1.45)* $p = 0.031$	0.47 (−0.20, 1.14) $p = 0.17$
BMI & Smoking (95% CI)	–	−0.04 (−0.72, 0.63) $p = 0.90$	0.68 (−0.01, 1.36) $p = 0.052$	0.21 (−0.50, 0.92) $p = 0.56$
WBC count 10^9/L				
Unadjusted (95% CI)	–	0.51 (−0.80, 1.82) $p = 0.44$	0.96 (−0.36, 2.28) $p = 0.15$	2.08 (0.74, 3.42)** $p = 0.003$
BMI & Smoking (95% CI)	–	0.60 (−0.65, 1.84) $p = 0.34$	0.69 (−055, 1.93) $p = 0.27$	1.33 (0.01, 2.65)* $p = 0.048$
CIS				
Unadjusted (95% CI)	–	−0.27 (−0.62, 0.56) $p = 0.93$	0.54 (−0.05, 1.13) $p = 0.073$	0.94 (0.34, 1.54)** $p = 0.003$
BMI & Smoking (95% CI)	–	0.11 (−0.48, 0.69) $p = 0.72$	0.47 (−0.10, 1.04) $p = 0.11$	0.66 (0.05, 1.26)* $p = 0.034$

*ACE, Adverse childhood experiences; CI, Confidence interval; CIS, Composite inflammation score; MDD, Major depressive disorder; WBC, White blood cell. *indicates significant difference compared to the control group, p-value < 0.05; **p-value < 0.01.*

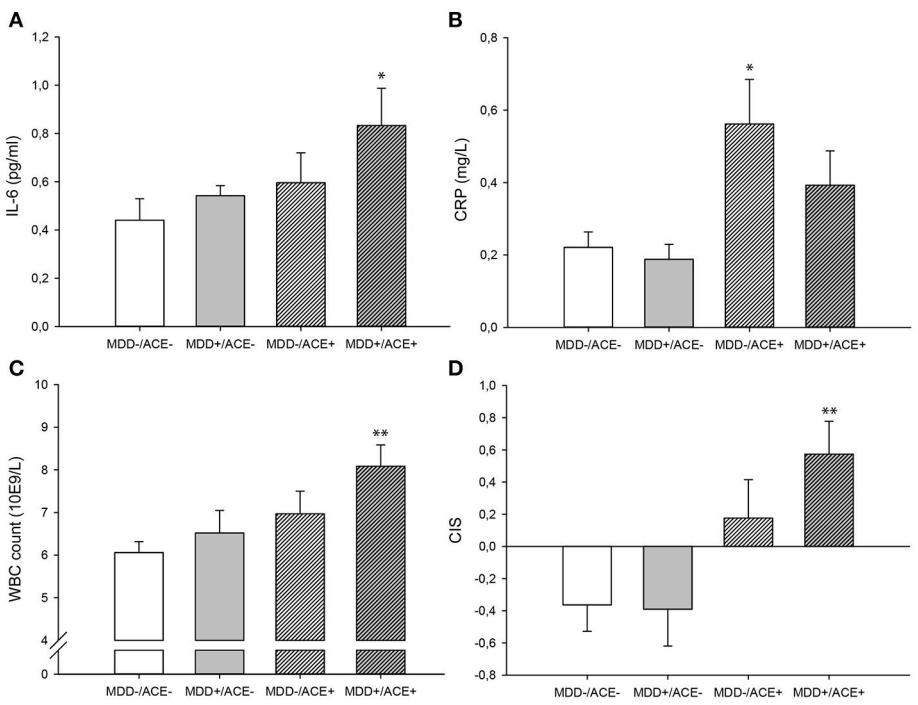

FIGURE 1 | Untransformed and unadjusted mean group values for **(A)** Il-6, **(B)** hsCRP, **(C)** WBC count and **(D)** the unadjusted mean group CIS (±SE). *p-value < 0.05, **p-value < 0.01 in comparison to the control group (MDD–/ACE–). ACE, Adverse childhood experiences; CIS, Composite inflammation score; MDD, Major depressive disorder; WBC, White blood cell.

Our study has several strengths. The study sample consisted of four carefully diagnosed groups which allowed us to disentangle the effects of MDD and ACE on inflammation. Furthermore, none of the patients and participants was taking psychotropic medications. All patients and participants received detailed diagnostics and a physical examination. Our findings of increased levels of inflammation in MDD patients (and to a lesser extent in healthy individuals) exposed to ACE, in this rather young study sample further emphasize the clinical importance of our results, since elevated inflammation is a risk factor implied in the development of somatic disorders like CVD (3, 42). Moreover, the pro-inflammatory state observed in depression also has consequences for treatment success, since patients with elevated inflammation are less likely to respond to conventional antidepressants (43, 44).

In summary, in this well-controlled study, we replicated findings from prior research suggesting accumulative effects of MDD and ACE on a more pro-inflammatory state, while inflammation levels did not differ between MDD patients without ACE and healthy controls. These findings suggest that a subgroup of MDD patients with a history of ACE might benefit from an anti-inflammatory intervention.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Charité's Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Charité's Ethics Committee.

AUTHOR CONTRIBUTIONS

KdP carried out the laboratory assays, participated in the data analyses, and drafted the manuscript. SE designed the study participated in the analysis and interpretation of study findings and provided editorial assistance. CH participated in the data analysis and provided editorial assistance. CD participated in the conduction of the study, interpretation of study findings and provided editorial assistance. CO participated in the study design, analysis and interpretation of study findings and provided editorial assistance. KW participated in the study design, analysis and interpretation of study findings and provided editorial assistance. LK conceived of and designed the study, conducted the study, participated in the analysis and interpretation of study findings, drafted portions of the manuscript and provided final editorial oversight.

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Hippo Signaling: Emerging Pathway in Stress-Related Psychiatric Disorders?

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Discovery of the Hippo pathway and its core components has made a significant impact on our progress in the understanding of organ development, tissue homeostasis, and regeneration. Upon diverse extracellular and intracellular stimuli, Hippo signaling regulates stemness, cell proliferation and apoptosis by a well-conserved signaling cascade, and disruption of these systems has been implicated in cancer as well as metabolic and neurodegenerative diseases. The central role of Hippo signaling in cell biology also results in prominent links to stress-regulated pathways. Genetic variations, epigenetically provoked upregulation of Hippo pathway members and dysregulation of cellular processes implicated in learning and memory, are linked to an increased risk of stress-related psychiatric disorders (SRPDs). In this review, we summarize recent findings, supporting the role of Hippo signaling in SRPDs by canonical and non-canonical Hippo pathway interactions.

Keywords: hippo pathway, KIBRA, psychophysiological stress, synaptic plasticity, glucocorticoids, GPCRs

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INTRODUCTION

When the Hippo pathway was first discovered in Drosophila, it appeared as a linear kinase cascade highly relevant for proliferation and homeostasis, because deletion of core component genes resulted in an uncontrolled growth of multiple tissues (1, 2). Subsequent research identified mammalian orthologs of Hippo components and additional kinases, transcription factors and various adapter proteins directly or indirectly involved in Hippo signaling, providing a complex molecular network with strong regulatory effects on development, homeostasis, and regeneration (3–5). Upstream activators of the Hippo pathway include G-protein-coupled receptors (GPCR), integrins, and cell-cell adhesion factors, stress-reactive glucocorticoid hormones, metabolism-regulating hormones, growth factors, and mitogens (6).

Dysregulated Hippo signaling is associated with various cancers and a wide range of metabolic, cardiovascular, neurodevelopmental, and neurodegenerative diseases (3, 7). Regulators of Hippo pathway are expressed in the adults' brain suggesting their implementation in normal brain performance. Recent research further extends the Hippo signaling network and its potential to be therapeutically harnessed based on genetic association studies linking Hippo pathway members to stress-related-psychiatric disorders (SRPDs) (8–11). Key molecular and cellular processes that are thought to be involved in the pathophysiology of SPRDs are modulated by Hippo pathway members. Furthermore, various proteins of the Hippo signaling pathway are linked via the GR, GPCRs, Wnt-signaling and other pathways to stress-regulated signaling cascades (12–16).

In this review we highlight emerging evidence of an interaction between Hippo signaling and the stress axis and suggest how this novel link may correlate with the genesis of SRPDs.

THE HIPPO PATHWAY IN MAMMALS AND ITS CANONICAL ACTIVATION

The regulatory endpoints of the Hippo pathway are the two homologous transcriptional co-activators, yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (3, 7) (**Figure 1**). YAP and TAZ are widely expressed throughout the brain and non-neuronal tissues especially during embryogenesis[1] In adult humans YAP is expressed in the subventricular zone of the lateral ventricle and subgranular zone of the dentate gyrus, the regions providing neurogenesis in mammalian brains. Weak immunostaining was found in the prefrontal cortex of humans (17). YAP expresses in the midbrain, possibly, to protect dopaminergic neurons from degeneration (18). TAZ expression appears to contribute to brain mitochondrial respiration, the function of hippocampal neurons and glia, and modulates cognitive abilities in mice (19). It is of note that Hippo pathway activity is retained in the adult hippocampus. A role of the hippocampus in neurogenesis and stress resilience (20), denote the Hippo pathway as a target for biomarker discovery and therapeutic interventions in SRPDs.

The regulation of YAP and TAZ is governed by two major protein kinase complexes, the mammalian Sterile 20-like kinases 1 and 2 (MST1/2), and the large tumor suppressor homolog LATS1/2 and their direct interaction partners SAV1 (MST1/2) and MOB1A/MOB1B (LATS1/2). Activation of MST1/2 and LATS1/2 causes phosphorylation of YAP/TAZ. Phospho-YAP/TAZ is either degraded or sequestered in the cytoplasm by the 14-3-3 protein, whereas after inactivation of the upstream kinase cascade dephosphorylated YAP/TAZ translocate to the nucleus. AJUBA antagonizes YAP phosphorylation and therefore prevents its activation. Through association with various transcription factors, like the TEAD family transcription factors (TEAD1-4), YAP/TAZ initiates transcription of several genes mainly involved in the regulation of development, homeostasis, and regeneration (3, 7) (**Figure 1**). This core-signaling cascade is activated/ inactivated by multiple stimuli and modulated by various post-translational modifications or through hetero complex re-organization, e.g., NF2 (Merlin) inhibits LATS through phosphorylation (3, 7) (**Figure 1**). Although, YAP and TAZ are primarily controlled at the level of their nuclear accumulation (nucleocytoplasmic shuttling), it is incompletely elucidated if nuclear entry occurs passively (diffusion), if it is a mediated process, or a combination of both (21). In a recent report mechanical forces have been shown to increase the permeability of the nuclear pore thereby facilitating the nuclear accumulation of YAP (22), whereas another study identified a nuclear localization sequence (NLS) and a nuclear export sequence (NES) for TAZ (21). Moreover 14-3-3 protein and TEAD family members have been proposed to be cytosolic and nuclear "retention factors," respectively (21).

[1]Mouse Brain Atlas. http://mousebrain.org/genesearch.html

NON-CANONICAL REGULATION OF HIPPO SIGNALING BY PSYCHOPHYSIOLOGICAL STRESS

Accumlating evidence suggests that the core complexes and accessory proteins of the Hippo pathway can be modulated by molecular pathways that play a fundamental role in stress signaling. The non-canonical regulation of the Hippo pathway with regard to SRPDs will be the focus of the following chapter.

GLUCOCORTICOIDS IMPACT ON HIPPO PATHWAY

Release of glucocorticoids (GCs), such as cortisol, from the adrenal glands, is the final stage of hypothalamic-pituitary-adrenal (HPA) axis activation during emotionally stressful experiences [psychophysiological stress, depicted as "stress" throughout the manuscript, (23)]. GCs belong to the class of steroid hormones and act via specialized nuclear receptors to adapt behavior to a constantly changing environment. Despite the critical role that stress plays for body homeostasis, it is widely implicated in the onset of SRPDs (23). Sorrentino and colleagues described a molecular cascade that links glucocorticoid signaling to YAP regulation. In an interdisciplinary approach the researchers show, that the activation of glucocorticoid receptors (GRs) results in elevated YAP protein levels, its translocation to the nucleus and subsequently to enhanced transcriptional activity. Fibronectin was identified as a target of the GR. Increased fibronectin expression stimulates the focal adhesion-Src pathway, which in turn activates cytoskeleton-dependent YAP activation providing a direct link between the stress-hormone axis and Hippo signaling (24).

GPCRs AND HIPPO PATHWAY IN SRPDs AND RELATED PSYCHOPATHOLOGIES

GPCR Signaling

Extracellular signals act on synapses to drive spine morphogenesis and synaptic plasticity. Among multiple classes of receptors G protein-coupled receptors (GPCRs) are the working horses of neuronal communication. Overexpression or exogenous stimulation of a variety of GPCRs corresponds to Hippo pathway activity. Serotonin 5-HT4, adrenergic α1B, metabotropic glutamate mGlu2, and adenosine A1A receptors are directly mediating neuronal transmission in the brain and are shown to contribute to stress-related abnormalities in mammals (25–29). These receptors, which are linked to brain-body crosstalk (LPA receptors, purinergic receptors, muscarinic acetylcholine receptor M1, angiontensin II receptor, free fatty acid receptor 1, platelet-activating factor receptor, thromboxane A2, frizzled homolog D4, complement component 3a receptor 1, estrogen receptor 1, opioid receptor Δ1, secretin receptor, thyroid-stimulating hormone receptor, gastrin-releasing peptide

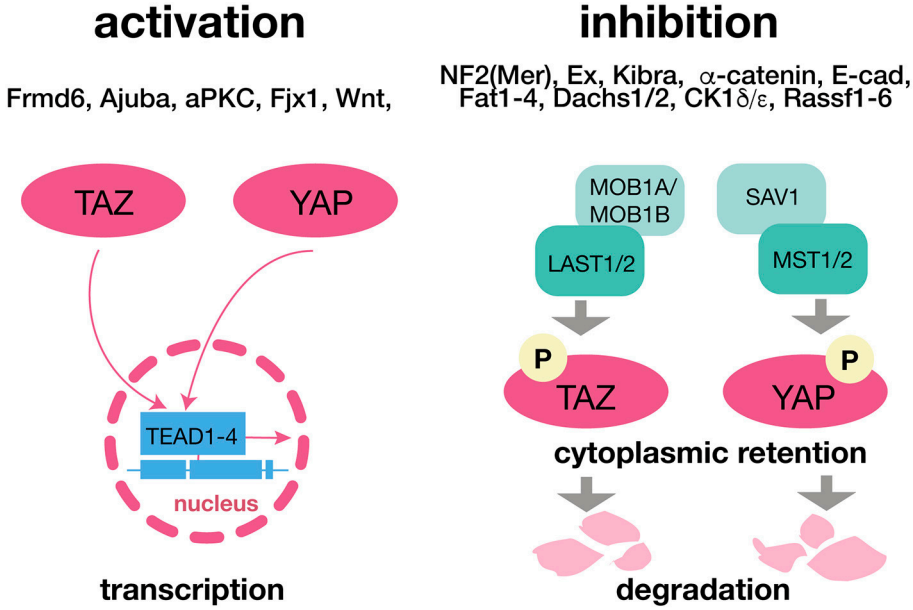

FIGURE 1 | The Hippo Pathway and its canonical upstream regulators. The translocation of YAP and TAZ and respective translational effects of the Hippo pathway are omitted when these two factors are phosphorylated due to LAST1/2 and MST1/2 activity. Both components can be affected independently via a wide range of canonical upsream regulators. Being retained in the cytoplasm YAP and TAZ are ubiquitinated and degraded. ADHD, attention deficit hyperactivity disorder. LATS 1/2, large tumor suppressor kinase 1/2; MOB1A/MOB1B, Mps one binder kinase activator 1A/1B; MST1/2, macrophage-stimulating protein 1/2; SAV1, salvador family WW domain containing protein 1 (protein WW45); TAZ, transcriptional co-activator with PDZ-binding motif; TEAD1-4, TEA domain transcription factors 1-4; YAP, yes-associated protein.

receptor, melanocortin receptor 1, somatostatin receptor 1, prostaglandin E receptor 2, and bombesin-like receptor 3) affect both the Hippo-YAP and Hippo-TAZ signaling via activation of Rho GTPases (16).

In contrast, dopamine D1 and adrenergic β2 receptors appear as a way for the selective inhibition of Hippo-YAP signaling. These GPCRs induce YAP phosphorylation mainly via cAMP and PKA (16).

Selective regulation of the Hippo-YAP signaling by 5-HT2B receptors activation has been shown in hepatocytes (30) and cardiomyocytes (31). These data suggest an effect of acute and chronic serotonin neurotransmission disturbance on Hippo signaling and provides a strong link between stress and related pathologies in peripheral organs. Although most available drugs to treat the symptoms of SRPDs (antidepressants) target serotonergic neurotransmission (32), a putative modulation of Hippo signaling by antidepressants remains a topic of future research.

Wnt SIGNALING

Components of the Wnt pathway are transcriptional targets and therefore downstream targets for the Hippo pathway (13–15). The upstream influence of the canonical Wnt/β-catenin signaling on the Hippo pathway has been described recently (12). Consequently, a dynamical interaction in the presence of Wnt YAP/TAZ is released from the destruction complex, escaping degradation in the cytoplasm. In absence of Wnt the YAP/TAZ-dependent β-TrCP (β-transducin repeats-containing proteins) recruitment allows β-catenin destruction (33). Notably, the β-TrCP-mediated β-catenin degradation is GSK3–dependent (34). GSK3 plays a critical role in the regulation of Wnt—Hippo interaction (14).

CANONICAL HIPPO PATHWAY LINKS TO SRPDs

Multiple studies have shown a direct association between members of the hippo pathway and SRPDs. Most data comes from genetic studies that report an association of allelic variation in the KIBRA (KIdney and BRAin) gene with (episodic) memory performance, gray and white matter volume and differences in functional brain activity (35–41). Substitution of C for T in the 9th intron (rs17070145) of the KIBRA gene, was first linked to memory performance and functional brain activity in a genome-wide association study (35). However, the functional role of the gene is still unclear since replication of the first results has proven difficult and sometimes delivered contradicting results. In line with the initial results, the rs17070145-T allele has been associated with better episodic memory functioning (36–41). However, several other studies have either associated the absence of rs17070145-T with better memory performance (42, 43), or were unable to show any link of this Single Nucleotide Polymorphism (SNP) with cognitive capabilities (43–46). CLSTN2 (calsyntenin 2), another hippo pathway member (SNP rs6439886), is mainly localized in the postsynaptic compartment of excitatory neurons in brain regions

relevant for learning and memory like the medial temporal lobe (47), and has also been linked to memory performance by Passotiropoulos et al. (35) and in subsequent cohorts (48, 49). Another study, however, did not support the influence of the KIBRA SNP, with or without the CLSTN2 SNP, on longitudinal memory decline or hippocampal atrophy in older adults (44).

It has been speculated, that the lack of consensus across studies stem from age-related neuropathological changes on memory performance, which may interact with polymorphisms such as KIBRA and CLSTN2, the so-called "resource modulation hypothesis" (40, 44). Supporting evidence comes from studies taking age, increased risk for specific diseases and pre-existing diseases into account (9, 10, 46). Stickel et al. (40) report, that KIBRA results in decreased verbal memory performance and lower brain volumes in CC homozygotes compared to T carriers, particularly among older persons (40). In individuals with unipolar depression, Pantzar et al. (10) showed an interactive effect of KIBRA and CLSTN2 polymorphisms on memory performance, but not in older individuals without depression (10). They also found poorer episodic recall and recognition performance in non-T carriers (10). In contrast, in patients with major depressive disorder, Liu et al. (9) found that rs17070145 associates with better memory performance in non-T carriers (9). In cognitively normal adults with different genetic risk of Alzheimer's disease, based on their Aβ-amyloid levels and apolipoprotein E (APOE) ε2/ε3/ε4 genotype, Porter et al. (46) reported faster rates of cognitive decline and hippocampal atrophy in individuals with higher Aβ-amyloid levels and APOE ε4 + ve, that did not carry the rs17070145-T allele (46). Although this suggests that the exact role of the KIBRA, SNP rs17070145 in learning and memory is still unclear, further investment in understanding its well-established role in cognitive performance is essential to make progress from mechanism to disease in SRPDs.

Another association of two neighboring SNPs in the KIBRA gene in almost complete linkage disequilibrium, rs10038727, and rs4576167, with lifetime risk for post-traumatic stress disorder was described in two samples from African conflict regions (8). Carriers of the minor allele of both SNPs displayed a diminished disease risk (8). Nitric oxide synthase 1 adaptor

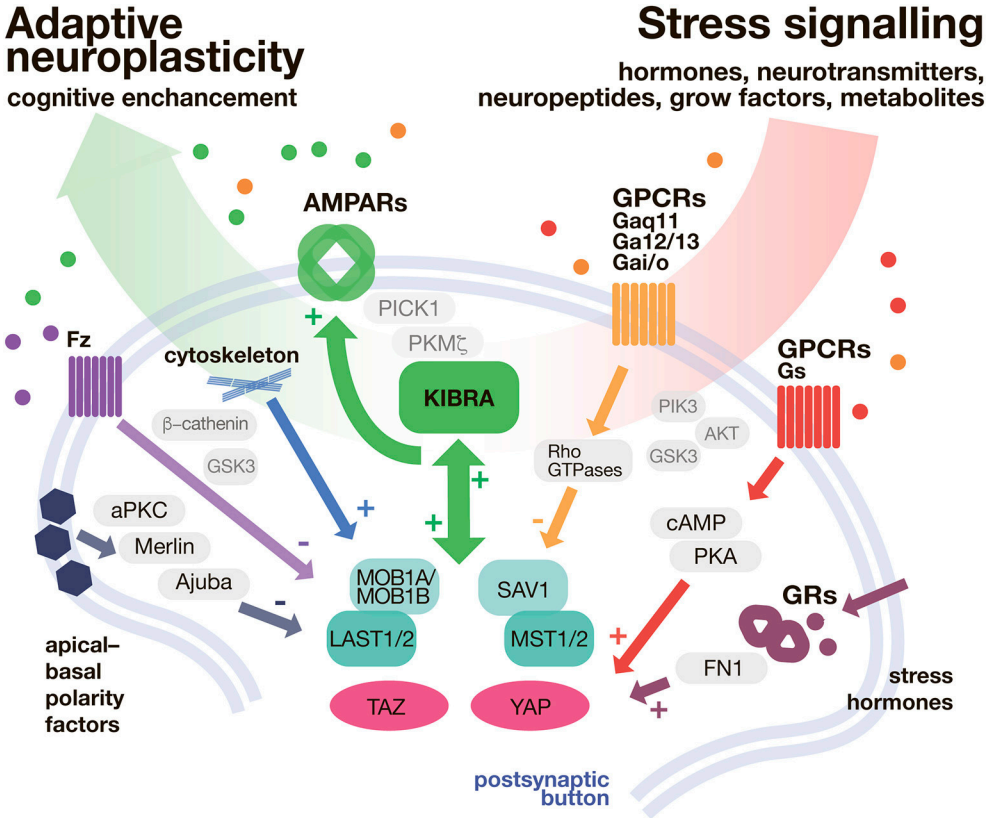

FIGURE 2 | KIBRA/Hippo pathway as a shunt of stressful input. In the postsynaptic button Hippo pathway is modulated with a variety of secondary messenger systems, implemented in transduction of neurontransmitters, neuropeptides, and hormones. The Hippo pathway biderectioinally interacts with KIBRA signaling. In turn, the adaptive, neuroplasticity determined by AMPAR expression (and trafficking) is promoted. AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; cAMP, cyclic adenosine monophosphate; FN1, fibronectin 1; Fz, Frizzeled; GSK3, Glycogen synthase kinase 3; GPCR, G protein coupled receptors; GR, glucocorticoid receptor; KIBRA, Kidney and Brain Protein 1 (also WWC1); LATS 1/2, large tumor suppressor kinase 1/2; MOB1A/MOB1B, Mps one binder kinase activator 1A/1B; MST1/2, macrophage-stimulating protein 1/2; PICK1, protein interacting with C-kinase 1; PKA, protein kinase A; PKC, protein kinase C; Rho GTPase, Rho guanosine-5′-triphosphatase; SAV1, salvador family WW domain containing protein 1 (protein WW45); TAZ, transcriptional co-activator with PDZ-binding motif; YAP, yes-associated protein.

protein (NOS1AP) also known as carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein (CAPON) is an adaptor protein of the Hippo pathway and is encoded by the NOS1AP gene in humans (11, 50). CAPON is supposed to modulate glutamate neurotransmission via interaction with postsynaptic density (PSD) scaffolding proteins PSD93 and PSD95 (50). Xu et al. (11) showed an increased expression of CAPON in the prefrontal cortex in post-mortem tissue of patients with bipolar disorder (11).

KIBRA AS POTENTIAL MEDIATOR OF SYNAPTIC STRESS EFFECTS

Accumulating evidence suggests that the scaffold protein expressed by the KIdney and BRAin gene [KIBRA; sometimes referred to as WW and C2 domain-containing protein 1 (WWC1)], is critical for synaptic plasticity, the cellular mechanism thought to underlie learning and memory (51–56). Althought it has not yet been demonstrated directly, KIBRA is a potential candidate to, at least partially, mediate the well-established stress effects on synaptic plasticity and cognitive performance (57, 58).

KIBRA is predominantly expressed in the kidney and the brain, in particular in structures important for learning and memory like the hippocampus, cortex, cerebellum, and hypothalamus (59, 60). In neuronal cells, KIBRA has a somatodendritic staining pattern with enrichment in perinuclear regions and the postsynaptic density (PSD) (54, 59). Previous studies have shown that KIBRA has various bindings partners, mainly mediated by the two N-terminal WW domains, a glutamic acid–rich motif and motifs for binding atypical PKC and PDZ domains (54, 56, 61). This includes the postsynaptic proteins dendrin and synaptopodin, postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs, the main fast stimulatory receptor of the neurotransmitter glutamate), and the atypical protein kinase C (PKC) isoform protein kinase Mζ (PKMζ) (52, 53, 59, 60, 62, 63).

PKMζ is brain specific and crucially involved in AMPA-receptor trafficking, a core mechanism of synaptic plasticity, and in the maintenance of long-term potentiation (LTP) in the hippocampus, which is thought to be the cellular correlate of learning and memory in mammals and involves AMPA and NMDA receptors of glutamate (51, 63–65). PKMζ is colocalized with KIBRA especially in the hippocampus and dentate gyrus (65), and KIBRA knock-out mice exhibit reduced learning and memory performance in spatial memory tasks, accompanied by decreased PKMζ levels (56). These results are in line with the observation that KIBRA associates with AMPARs and its partner protein interacting with C-kinase 1 (PICK1), which has been shown to accelerate the rate of AMPAR subunit recycling to the postsynaptic membrane (53). Moreover, KIBRA knock out mice exhibit an impaired LTP and long-term depression (LTD) in the hippocampus and show deficits in contextual fear learning and memory (53).

Overexpression of KIBRA in neurons facilitates LTP, but prevents the induction of LTD, likely by an increased constitutive recycling of AMPARs. In contrast, knock down of KIBRA abolishes LTP and decreases AMPAR recycling supporting a role of KIBRA as a bidirectional regulator of synaptic plasticity in hippocampal neurons (52). In a recent study, Tracy et al. (55) show that memory loss and LTP impairment in a mouse model of Alzheimer's disease critically depends on reduced synaptic KIBRA levels accompanied by reduced activity-induced postsynaptic actin remodeling and AMPAR insertion, which can be rescued by promoting actin polymerization or by restoring KIBRA expression (55).

The WWC family comprises two additional highly similar paralogs, WWC2, and WWC3 (61). Although it has been speculated that WWC2 can balance WWC1 knock out (53), their role in brain function remains unclear.

CONCLUSION

Strong evidence suggests that both, Hippo- and stress signaling are involved in the pathophysiology of SRPDs. However, the possible interaction between Hippo signaling and the stress hormone axis has been widely neglected so far. Especially KIBRA as a mediator of adaptive neuroplasticity that is directly linked to the stress hormone axis via GR-signaling might balance the reduced cognitive capabilities observed in most SRPDs (see **Figure 2**).

Although there are many important questions that remain unanswered (e.g., exact role of KIBRA in memory), pharmacological targeting of Hippo signaling might offer guidance for the development of novel prophylactic and therapeutic approaches to treat SRPDs more effectively.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Childhood Adversity Is Associated With Increased *KITLG* Methylation in Healthy Individuals but Not in Bipolar Disorder Patients

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Background: Childhood adversity increases the risk of a range of mental disorders including bipolar disorder, but the underlying mechanisms are still unknown. Previous studies identified DNA methylation levels at the cg27512205 locus on the KIT Ligand (*KITLG*) gene as a mediator between childhood adversity and stress responsivity. This raises the question whether this locus also plays a role in stress related disorders such as bipolar disorder. Therefore, the current study aims to compare the level of *KITLG* (cg27512205) methylation between bipolar patients and healthy individuals and its relation to childhood adversity.

Methods: *KITLG* (cg27512205) methylation was measured in 50 bipolar disorder patients and 91 healthy control participants using the HumanMethylation450K BeadChip platform. Childhood adversity in each individual was assessed using the Childhood Trauma Questionnaire. Analyses of the association of *KITLG* methylation with bipolar disorder, the association of childhood adversity with bipolar disorder as well as the association of *KITLG* methylation with childhood adversity in bipolar patients and controls were conducted using linear regression with age, gender, childhood adversity, smoking, and cell-type composition estimates as covariates.

Results: *KITLG* (cg27512205) methylation level was significantly lower in bipolar disorder patients ($\beta = -0.351$, $t = -6.316$ $p < 0.001$). Childhood adversity levels were significantly higher in the bipolar disorder group ($\beta = 4.903$, $t = 2.99$, $p = 0.003$). In the bipolar disorder patients *KITLG* methylation was not associated with childhood adversity ($\beta = 0.004$, $t = 1.039$, $p = 0.304$) in contrast to the healthy controls ($\beta = 0.012$, $t = 3.15$, $p = 0.002$).

Conclusions: *KITLG* methylation was lower in bipolar disorder despite high levels of childhood adversity, whereas childhood adversity was associated with higher *KITLG* methylation in healthy controls. In addition to lower methylation at this locus there is an indication that failure to adjust *KITLG* methylation to high levels of childhood adversity is a risk factor for bipolar disorder.

Keywords: DNA methylation, *KITLG*, bipolar disorder, childhood adversity, stress

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INTRODUCTION

Bipolar disorder is a severe psychiatric disorder characterized by mood episodes ranging from mania to severe depression (1). The life time prevalence of bipolar disorder is 0.5–1.5% in the general population and 5–10% for first degree relatives (2). Although the pathogenesis of bipolar disorder is not well understood, both genetic and environment factors are involved.

One major detrimental environmental factor for developing mental disorders including bipolar disorder later in life is childhood adversity (3, 4). Childhood adversity encompasses a wild range of adversities before the age of 16, such as physical, emotional and sexual abuse, household poverty, separation from a parent and neglect. Previous studies found that children with childhood adversity have a high risk to develop bipolar disorder (5). However, how childhood adversity contributes to the development of bipolar disorder is still largely unknown.

Recent studies highlight the role of DNA methylation in the pathway of childhood adversity to bipolar disorder (6). DNA methylation is one of the epigenetic mechanisms that can modulate gene expression in response to the environment might account for part of the risk to bipolar disorder (7). Childhood adversity as a detrimental environmental factor could therefore, contribute to DNA methylation differences in key pathways involved in bipolar disorder. In our previous genome-wide DNA methylation analysis, KIT Ligand (*KITLG*) (cg27512205) methylation was positively associated with childhood trauma and served as a mediator between childhood trauma and blunted cortisol stress reactivity in healthy controls (8). Since impaired cortisol stress reactivity is associated with bipolar disorder (9, 10), this could imply an association between *KITLG* methylation with bipolar disorder. Moreover, bipolar disorder patients also report higher levels of childhood adversity (11), which may lead to higher *KITLG* methylation if the previous findings in healthy controls were to be extrapolated to bipolar disorder patients.

Therefore, the current study hypothesizes the presence of higher *KITLG* methylation in bipolar disorder patients as compared to healthy controls in agreement with expected higher level of childhood adversity. To examine this hypothesis, we investigate the relationship between *KITLG* (cg27512205) methylation level in a case-control sample of bipolar disorder patients and healthy controls and the relation to childhood adversity.

MATERIALS AND METHODS

Study Population

Sample recruitment has been previously described (8, 12). In short, 50 bipolar patients and 91 control participants were included at the University Medical Center Utrecht (UMCU). All participants had three or more Dutch grandparents. All participants provided informed consent prior to the inclusion of the study, and the study was approved by the Medical Ethics Committee of the UMCU and performed according to the ICH guidelines for Good Clinical Practice and the latest amendments of the Declaration of Helsinki. All the blood samples from the participants were drawn in the morning before 12 a.m. None of the healthy controls were taking any prescription medication at the time of testing nor did any of the participants ever participate in stress-related research before. To verify drug use, first self-report of current use of psychoactive substances was obtained followed by checking with urine multi-drug screening device (InstantView). If participants smoked daily, they were defined as a smoker. Confirmation of the absence of any mental or physical disorder in the healthy controls was obtained by an independent rater in an interview according to the Mini-International Neuropsychiatric Interview (MINI) plus criteria (13). For bipolar disorder participants only, the Structured Clinical Interview for DSM-IV (SCID) was used to diagnose the clinical characteristics, including mood and psychotic symptoms, number of manic, and depressive episodes, comorbid psychiatric diagnosis and age of disease onset (14). Euthymia in the bipolar disorder patients was established using the Inventory for Depressive Symptoms—Self Report (IDS-SR) (15) and manic symptoms were assessed using the Altman Self-Rating Mania Scale (ASRM) (16). All patients were on a stable (at least 1 month) medication dose. The sample characteristics are provided in **Table 1**.

Childhood Adversity

Childhood adversity was measured using the short version of the Childhood Trauma Questionnaire (CTQ) (17). The Dutch translation of CTQ and validity of the 25 clinical CTQ items has been demonstrated in clinical and population samples (17, 18). One translation item "I believe I was molested" was excluded since this translation was found to be an invalid indicator of childhood sexual abuse in a previous validation study (18). We calculate the sum score of all individual abuse questions to generate a continuous outcome.

DNA Methylation Analyses

DNA methylation level of *KITLG* (cg27512205) was extracted from previously described Illumina Infinium HumanMethylation450K BeadChip data (12). In short, DNA

TABLE 1 | Sample characteristics ($n = 141$).

Variable n (%) or mean (range)	Control	Bipolar disorder	*p*
Number, n	91	50	
Age, years; mean (sd)	33.50 (15.68)	43.52 (12.83)	<0.001
Female sex, n (%)	44 (48.4%)	25 (50%)	0.853
Smoking, n (%)	11 (12.1%)	18 (36%)	0.001
Age at onset, years; mean (sd)	None	26.37 (11.45)	
Number of episodes; mean (sd)	None	6.39 (5.12)	
Childhood trauma score (mean, sd)	31.77 (8.37)	36.56 (10.28)	0.004
BIPOLAR DISORDER GROUP			
Bipolar I, n	None	46	
Bipolar II, n	None	4	
Bipolar disorder Not Otherwise Specified (NOS), n	None	0	

was obtained from blood using a commercial kit (Qiagen, CA, USA). The DNA concentration and integrity were assessed by riboGreen and BioAnalyser, respectively. Bisulfite conversion was performed by using Zymo Kit (ZYMO Research, CA, USA). Samples were distributed on different chips based on gender and age to reduce batch effects. To remove further systematic differences, the samples were normalized using Beta MIxture Quantile dilation (BMIQ) and batch effects of sentrix array and position were removed with the Combat procedure from the sva package (19). Intensity and quality parameters were obtained from genome studio software. X chromosome, Y chromosome and non-specific binding probes were removed (20). Based on literature (21), probes were excluded based on a detection P value > 0.001 and bead count <5 in 5% of the samples. In addition, probes with SNPs of minor allele frequency $>5\%$ within 10 base pairs of the primer were excluded after constructing ancestry estimates as proposed by Barfield et al. (22). 385,882 DNA methylation probes survived quality control, including the *KITLG* cg27512205 probe. All samples were included as none of the samples had more than 1% of probes failed. Cell-type composition estimates were derived using the Houseman procedure (23). Methylation analyses were carried out using M-values (log2 ratio of methylation probe intensity) for better statistical validity (24), but beta values of methylation were used for graphical display.

Statistical Analysis

Quality control of DNA methylation was conducted with R version 3.1.2 (25). Other statistical analyses were performed using SPSS Statistics 23.0. Analysis of the association of *KITLG* (cg27512205) methylation with bipolar disorder was done using linear regression with *KITLG* methylation as dependent and diagnosis as the main determinant. Age, gender, childhood adversity, smoking, and six different cell-type composition estimates (B cells, CD8 T cells, CD4 T cells, natural killer cells, monocytes, and granulocytes) were included as covariates since they have a potential impact on DNA methylation (26). Differences in childhood adversity between patients and controls were examined using linear regression, in a separate model. This relation was analyzed while adjusted for age, gender and smoking status. The association between *KITLG* methylation and childhood adversity was analyzed by linear regression model in control and bipolar patients separately. Age, gender and smoking were included as covariates.

RESULTS

Baseline Characteristics of Bipolar Cohort

A summary of the sample characteristics of bipolar disorder cohort is provided in **Table 1.** In the bipolar disorder group, 46 participants were diagnosed with bipolar disorder I type and 4 with bipolar disorder II type. The mean age of participants in the control group was significantly lower than in the bipolar disorder (BD) group (control = 33.5, BD = 43.52, $p < 0.001$). The proportion of smokers was significantly higher in the BD group (control = 12.1%, BD = 36%, $P = 0.001$), but no relation was present between *KITLG* methylation and smoking status ($\beta = 0.001$, $t = 0.099$, $p = 0.922$). Childhood trauma score was significantly higher in bipolar group than in controls ($\beta = 4.903$, $t = 2.990$, $p = 0.003$; model fit: $F = 8.940$, $p = 0.003$, $R^2 = 0.060$), but these differences were attenuated after adjustment for age gender and smoking($\beta = 3.043$, $t = 1.817$, $p = 0.071$; model fit: $F = 8.498$, $p < 0.001$, $R^2 = 0.200$). In the bipolar disorder group, comorbid psychiatric diagnosis were: Anxiety disorder Not Otherwise Specified (NOS) ($n = 1$), Generalized anxiety disorder ($n = 2$), Panic disorders ($n = 4$); Agoraphobia without history of panic disorder ($n = 1$), Specific phobia ($n = 2$), Obsessive-compulsive disorder ($n = 2$), Posttraumatic stress disorder ($n = 1$). Considering to the low frequency of the comorbid psychiatric diagnosis in the bipolar disorder group, we do not specifically exam the association of each comorbid psychiatric diagnosis with *KITLG* methylation level.

KITLG Methylation Analyses

KITLG methylation level was significantly lower in bipolar disorder patients compared to the healthy controls (mean control = 0.185, mean bipolar = 0.139) ($\beta = -0.351$, $t = -6.316$ $p < 0.001$; model fit: $F = 18.56$, $p < 0.001$, $R^2 = 0.407$) after adjustment for age, gender, childhood adversity, smoking, and cell types. **Figure 1** shows the adjusted individual levels of *KITLG* methylation per diagnostic group. No association of medication (mood stabilizer, antidepressant and antipsychotics) with *KITLG* methylation was present in the bipolar disorder group: Mood-stabilizers ($\beta = 0.008$, $t = 1.153$, $p = 0.255$); antidepressants ($\beta = 0.006$, $t = 0.732$, $p = 0.468$) and antipsychotics ($\beta = -0.008$, $t = -1.279$, $p = 0.208$), (Model fit: $F = 0.937$, $p = 0.488$, $R^2 = 0.135$).

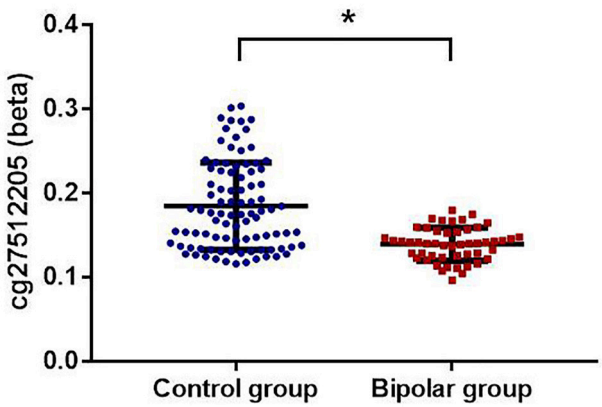

FIGURE 1 | KITLG (cg27512205) methylation (beta value) in healthy controls (blue dots) and bipolar disorder patients (red square). Black bar on each column shows the standard deviation of beta value of KITLG methylation in each group. Mean methylation level of KITLG is significantly lower in the bipolar group (*$p < 0.001$).

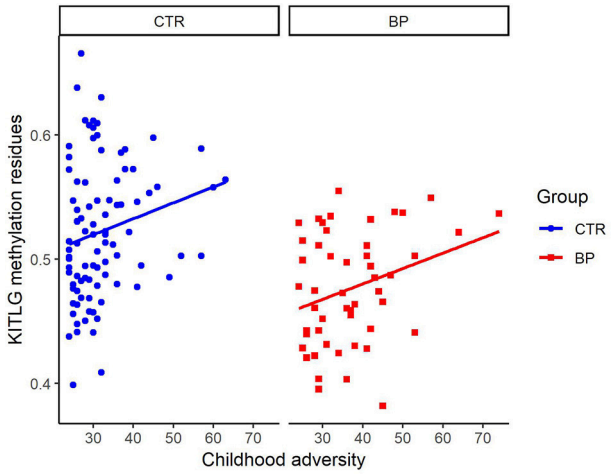

FIGURE 2 | Association of KITLG (cg26512205) methylation level with childhood adversity in both healthy controls (blue dots) and bipolar disorder patients (red square). Y-axis is the beta value of KITLG (cg26512205) methylation level after adjustment for cell type composition, age, gender and smoking. X-axis is the childhood adversity score. Significant positive association between childhood adversity and KITLG methylation was present in the healthy individuals (b = 0.012, p = 0.002). No significant association between KITLG methylation and childhood adversity in the bipolar disorder patients was present (b = 0.004, t = 1.039, p = 0.304).

Association Between Childhood Adversity and KITLG Methylation

Figure 2 shows the association of *KITLG* (cg26512205) methylation level (beta value) with childhood adversity in both healthy controls and bipolar disorder patients. There was no significant association between *KITLG* methylation and childhood adversity in the bipolar disorder patients ($\beta = 0.004$, $t = 1.039$, $p = 0.304$), whereas there was a significant positive association between childhood adversity and *KITLG* methylation associated in the healthy individuals ($\beta = 0.012$, $p = 0.002$; model fit: $F = 23.11$, $p < 0.001$, $R^2 = 0.444$).

DISCUSSION

Here, we follow up the previously reported association of *KITLG* methylation with childhood adversity and stress reactivity by exploring the relationship between *KITLG* DNA methylation levels at the locus cg27512205 and bipolar disorder. To our knowledge, this is the first study to report the association of *KITLG* methylation with bipolar disorder. We found lower DNA methylation levels at this stress related gene in bipolar disorder patients ($n = 50$) than in healthy controls ($n = 91$). In contrast to the positive association between childhood adversity with *KITLG* methylation in controls, we did not observe such an association in bipolar disorder patients. These findings suggest that failure to increase *KITLG* methylation in response to childhood adversity may constitute a risk factor for bipolar disorder.

Previously, we already reported of the positive association between *KITLG* methylation and childhood adversity in healthy controls (8). It is this finding that led to the expectation of *KITLG* hypermethylation among bipolar disorder patients exposed to higher levels of childhood adversity. However, the current study found *KITLG* hypomethylation in bipolar disorder patients and no relationship between childhood adversity and *KITLG* methylation in this group. This finding is consistent with a model whereby *KITLG* hypermethylation after childhood adversity is adaptive and failure to adapt is a characteristic of bipolar disorder patients. However, visual inspection of the relations between childhood adversity and *KITLG* methylation (**Figure 2**) points to systematic lower *KITLG* methylation in bipolar disorder.

Although unexpected, these findings are consistent with other recent reports that the protein coded by *KITLG* gene, known as stem cell factor (SCF), is significantly higher in children of bipolar disorder patients who develop mood disorder later in life (27). These higher levels of the *KITLG* protein SCF before disease onset are consistent with less repression on gene expression and transcription (28) and DNA hypomethylation at this locus. The specific *KITLG* locus (cg27512205, chr12: 88579621) that we focused on in the current study, is located in a H3K27ac-enriched region as well as on the 5' end of a CpG island near the *KITLG* gene. Mechanistically, DNA hypomethylation in the H3K27ac-enriched region is associated with a more open chromatin structure which indicates active gene transcription (29, 30). Moreover, DNA methylation differences frequently occur in CpG island shores and subsequently affect gene transcription and expression (31). These two co-occurrences suggest that *KITLG* hypomethylation at this CpG locus could indeed alter gene transcription and SCF levels. Another factor that could influence gene transcription level are genetic variants. For instance, gene polymorphism of *FKBP5*, an important functional regulator of the glucocorticoid receptor (GR), can mediated gene–childhood trauma interactions through DNA methylation level (32) and similarly genetic variants modify the methylation response to maternal famine (33). The *KITLG* locus in the current study contains just one genetic variant with no functional relevance for expression and therefore no indication of a role in genetic regulation is currently available.

A putative link between *KITLG* function and bipolar disorder is that the ligand of the C-kit receptor (SCF), is involved in hematopoiesis (34), neurogenesis, and neuroprotection (35) and induces glucocorticoid receptor gene (*NR3C1*) expression in response to stress induced erythropoiesis (36). This implies a positive regulation of *KITLG* gene to *NR3C1* expression, a key gene in the stress response (37, 38), that in term plays a role in bipolar disorder (9) and the response to trauma (39–41). Though the current finding is based on blood, the database from Hannon et al, shows that methylation of this specific *KITLG* locus (cg27512205) in the blood is significantly correlated with prefrontal cortex and superior temporal gyrus in the brain (42). This implies that blood *KITLG* methylation may serve as a proxy for *KITLG* methylation in these brain areas.

Some limitations need to be considered when interpreting these results. First, the focus on one specific locus (cg27512205)

based on our previous work, could potentially neglect DNA methylation at other genes that play a role in bipolar disorder. Using available Illumina Infinium HumanMethylation450K BeadChip data, an unbiased genome-wide DNA methylation analysis to investigate the interaction between bipolar disorder and childhood adversity may further our understanding of epigenetic difference related to childhood adversity and bipolar disorder. Second, though for some epigenetic loci blood may provide a reasonable proxy based on concordances in methylation patters between blood and brain (43, 44), it is a limitation considering that bipolar disorder is a psychiatry disorder residing largely in the brain. Another limitation of the study is that the Illumina 450 k BeadChip cannot distinguish between 5-Methylcytosine and 5-Hydroxymethylcytosin.

In conclusion, this study shows that *KITLG* methylation level is significantly lower in bipolar disorder despite relatively high childhood adversity exposure in bipolar disorder patients. This suggests a failure to adjust this epigenetic mark in response to childhood adversity in those vulnerable to bipolar disorder.

ETHICS STATEMENT

All subjects gave written informed consent before participation. This study was carried out in accordance with GCP guidelines and was approved by the Medical Ethics Committee of the UMCU.

AUTHOR CONTRIBUTIONS

MB and CV designed the study. LdW, MB, and CV collected the data. MB, LH, and YH performed statistical analysis. MB, CV, and YH wrote the manuscript. All authors read and approved the final manuscript.

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The Association of Childhood Maltreatment With Lipid Peroxidation and DNA Damage in Postpartum Women

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Childhood maltreatment (CM) is associated with an increased risk for the development of psychiatric and somatic disorders in later life. A potential link could be oxidative stress, which is defined as the imbalance between the amount of reactive oxygen species (ROS) and the neutralizing capacity of anti-oxidative defense systems. However, the findings linking CM with oxidative stress have been inconsistent so far. In this study, we aimed to further explore this association by investigating biological markers of DNA and lipid damage due to oxidation in a comprehensive approach over two study cohorts of postpartum women (study cohort I and study cohort II). The severity of CM experiences (maltreatment load) was assessed in both studies using the *Childhood Trauma Questionnaire*. In study cohort I ($N = 30$), we investigated whether CM was associated with higher levels of structural DNA damage in peripheral blood mononuclear cells (PBMC) by two methods that are highly sensitive for detecting nuclear DNA strand breaks (comet assay and γH2AX staining). In study cohort II ($N = 117$), we then assessed in a larger cohort, that was specifically controlled for potential confounders for oxidative stress measurements, two established serum and plasma biomarkers of oxidative stress, one representing oxidative DNA and RNA damage (8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanosine; 8-OH(d)G) and the other representing lipid peroxidation (8-isoprostane). In study cohort I, the analyses revealed no significant main effects of maltreatment load on cellular measures of nuclear DNA damage. The analyses of peripheral oxidative stress biomarkers in study cohort II revealed a significant main effect of maltreatment load on free 8-isoprostane plasma levels, but not on total 8-isprostane plasma levels and 8-OH(d)G serum levels. Taken together, by combining different methods and two study cohorts, we found no indications for higher oxidative DNA damages with higher maltreatment load in postpartum women. Further research is needed to investigate whether this increase in free 8-isoprostane is a marker for oxidative stress or whether it is instead functionally involved in ROS-related signaling pathways that potentially regulate inflammatory processes following a history of CM.

Keywords: childhood maltreatment, oxidative stress, lipid peroxidation, DNA damage, 8-isoprostane, 8-OH(d)G, comet assay, γH2AX

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INTRODUCTION

The experience of emotional, physical and/or sexual abuse, as well as emotional and/or physical neglect during childhood (i.e., childhood maltreatment [CM]) may cast a long shadow on adult health: A growing body of literature has shown that individuals with a history of CM are at greater risk to develop both mental and physical disorders later in life (1). Although accumulating research supports the hypothesis that CM is biologically embedded and exerts a long-lasting influence on stress-responsive systems (2, 3), research elucidating the causal pathways underlying the association of CM with adult health outcomes is nevertheless sparse. The research investigating this potential link focuses more and more on oxidative stress, which is defined as the imbalance between the amount of reactive oxygen species (ROS) and the neutralizing capacity of anti-oxidative defense systems (4).

Physiologically, ROS are produced in several subcellular structures (mainly within mitochondria) and serve important signaling functions that are essential for the coordination of metabolic, inflammatory, and stress response-related processes (4, 5). If the amount of ROS production, however, exceeds the physiological level and cannot be counterbalanced by the body's antioxidant defense systems, ROS readily attack lipids, proteins, DNA, and RNA (6). These ROS-induced modifications are also often applied in biomedical research as stable biomarkers to assess states of oxidative stress. One of the most investigated biomarkers for oxidative stress is 8-isoprostane, a specific peroxidation product of arachidonic acid and therefore a marker of lipid peroxidation (7). The levels of the oxidized nucleobase guanine within DNA (8-hydroxy-2′-deoxyguanosine; 8-OHdG) and RNA (8-hydroxyguanosine; 8-OHG) are often used as circulating markers for oxidative DNA and RNA damage (8). In addition, (oxidative) DNA damage can further be assessed on a cellular level by the comet assay or by the staining for phosphorylated histone H2AX (γH2AX). While the comet assay is a direct measure for DNA single and double strand breaks (9), γH2AX plays a role in signaling DNA double strand breaks and initiating their repair by supporting the recruitment and localization of DNA repair proteins (10). If such oxidative damages accumulate over time, they may have detrimental effects both at the cellular and at the systemic level (6, 11).

So far, oxidative stress was found to be involved in many physical diseases, like migraine, neurodegenerative diseases, cardiovascular diseases, and cancer (12–15)—amongst them several disorders that are observed at higher rates in individuals with a history of CM (1). Oxidative stress and related damages have also been implicated more and more in psychiatric disorders (16), including depression [see (17) for a review], bipolar disorder [see (18) for a meta-analysis], schizophrenia (19, 20), posttraumatic stress disorder (PTSD) (21), anxiety disorders (22), and different personality disorders (23). In affective disorders, higher oxidative stress and decreased antioxidant enzyme activities were associated with a lower health-related quality of life (24). Not only psychiatric disorders, but also other psychological stress factors like psychosocial stress (25), subjectively perceived stress (26, 27), chronic caregiving stress (27, 28), intimate partner violence (29), and sociodemographic disadvantage (30) were all reported to be associated with increased oxidative stress levels.

With regard to CM, findings have however been inconsistent so far: While do Prado et al. (31) reported that CM was associated with higher plasma levels of oxidative-stress-related protein carbonylation and an imbalance between oxidative molecules and antioxidants, Fanning et al. (23) found no significant association between CM and plasma levels of oxidative stress biomarkers (8-OH(d)G and 8-isoprostane) in individuals with different personality disorders. Additionally, Bergholz et al. (32) recently showed an association between complex childhood traumatization and nuclear DNA damage (γH2AX staining) in peripheral blood lymphocytes, while Simsek et al. (33) previously reported that children with a history of childhood sexual abuse did not differ in serum levels of antioxidant enzymes, the antioxidant coenzyme Q, and DNA damage (8-OH(d)G) from children without such experiences.

By investigating risk and resilience factors in the transgenerational transmission of CM in two study cohorts of postpartum women (study cohort I and study cohort II), we also found evidence for alterations in serum oxidative stress biomarkers and serum antioxidants applying targeted (study cohort I) and untargeted (study cohort II) metabolomics analyses (34, 35). Study cohort I showed reduced serum levels of metabolites with antioxidant capacity (L-carnitine and acetylcarnitine) and increased biomarkers of oxidative stress (Arginine-to-Citrulline ratio) (34). In study cohort II, untargeted metabolomics indicated higher serum levels of bilirubin IXa, another metabolite with antioxidant capacity, among women with CM compared to non-exposed women (35). Bilirubin is an end product of heme degradation by heme oxygenase-1 (HO-1), an enzyme with known anti-inflammatory and anti-oxidative properties (36, 37). Accordingly, higher levels of serum bilirubin were previously suggested to reflect the intensity of initial oxidative stress (38). Further analyses in study cohort I investigating the respiratory activity of mitochondria—the main producers of ROS—in intact peripheral blood mononuclear cells (PBMC), showed that CM was not only associated with alterations in mitochondrial activity, but also indicated an increase in cellular ROS production with increasing severity of CM experiences (34). These measures were further associated with a pro-inflammatory status of PBMC as represented by an increased spontaneous release of pro-inflammatory cytokines (34). As mitochondria and ROS are critical regulators of inflammatory processes (11, 39, 40), these findings suggest that alterations in mitochondrial activity and ROS production might not only constitute stress-related cellular damages but could also be functionally involved in adaptive signaling pathways. In the same study cohort, we observed that telomeres, the

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; BMI, body mass index; CI, confidence interval; CM, childhood maltreatment; CRP, C-reactive protein; CTQ, *Childhood Trauma Questionnaire*; HO-1, heme oxygenase-1; PBMC, peripheral blood mononuclear cells; PIM, probabilistic index model; PTSD, posttraumatic stress disorder; ROS, reactive oxygen species; SCID, Structured Clinical Interview; γH2AX, phosphorylated histone H2AX.

protective caps of our chromosomes that are more vulnerable for oxidative DNA damages than the rest of the genome (41), were significantly shorter in the long-living immune cell subset of memory cytotoxic T cells in women with CM compared to those without (42).

In sum, the complex picture arising on CM-related changes in ROS levels and the question whether oxidative stress is of physiological importance with regard to its signaling function or has damaging effects, remains far from being understood. Continuing our previous analyses in study cohort I (34) and study cohort II (35), this study aimed to investigate markers of oxidative DNA and lipid damage in a comprehensive approach over these two study cohorts of postpartum women with CM. In study cohort I ($N = 30$), we investigated whether CM was associated with higher levels of oxidative DNA damage in PBMC by two methods that are highly sensitive for detecting nuclear DNA strand breaks (comet assay and γH2AX staining). In study cohort II ($N = 117$), we then assessed in this larger, independent study cohort, that was specifically controlled for potential confounders for oxidative stress measurements, two established blood serum and plasma biomarkers of oxidative stress, one representing oxidative DNA and RNA damage (8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanosine; 8-OH(d)G) and the other representing lipid peroxidation (8-isoprostane).

MATERIALS AND METHODS

Design and Procedure of Study Cohort I and Study Cohort II

Participants of two longitudinal studies (study cohort I and study cohort II; see Measures in study cohort I and Analyses in study cohort II for detailed description), both investigating risk and resilience factors in the transgenerational transmission of CM, were used for the analyses. Study cohort I constituted thereby the pilot study to show the feasibility for a large-scale assessment, i.e., study cohort II, which was part of the project "My Childhood—Your Childhood." For both studies, women were recruited shortly after giving birth to a child (<1 week postpartum) at the maternity ward of the Ulm University Hospital (time point t_0). Exclusion criteria for study participation were maternal age under 18 years, severe health problems of mother or child, severe complications during parturition, and an insufficient knowledge of the German language. Participating mother-infant-dyads were then accompanied over 1 year with two follow-up assessments, the first 3 months postpartum (t_1) and the second 12 months postpartum (t_2). The studies were approved by the Ethics Committee of Ulm University and all procedures followed the current version of the Declaration of Helsinki (43).

After providing written informed consent, women were retrospectively interviewed about their history of maltreatment experiences below the age of 18 years with the German short version of the *Childhood Trauma Questionnaire* (44–46). The CTQ covers the five CM subscales emotional, physical, and sexual abuse as well as emotional and physical neglect. The CTQ sum score (range 25–125) was used as a cumulative measure for the severity of maltreatment experiences, the so-called *maltreatment load* (47). Using standardized cut-off criteria for the classification of CM based on CTQ sum scores (44, 45), participants were categorized into "no CM," "low CM," "moderate CM," and "severe CM" based on reported CM experiences for recruitment, follow-up, and selection of study participants for biological analyses (see Study participants of study cohort I and Study participants of study cohort II). In addition to the assessment of CM experiences, women were further asked to provide basic socio-demographic information at t_0.

During the follow-up interview at t_1, women provided detailed socio-demographic, clinical, and medical data in self-report. Additionally, whole blood samples were collected by venipuncture between 11 a.m. and 2:30 p.m. for the isolation of PBMC, plasma, and serum samples (EDTA-Monovettes for plasma collection and for whole blood sampling for PBMC isolation as well as S-Monovettes for serum collection; Sarstedt, Nümbrecht, Germany). To minimize additional acute psychological strain, the study participants were not obligated to fast overnight prior to the assessment.

Serum C-Reactive Protein (CRP) Content

To exclude participants who presented with signs of an acute inflammatory status at t_1, we assessed the serum CRP levels in all participants of study cohort I and study cohort II. For serum collection, whole blood was centrifuged for 10 min at 3,000 g and 4°C. Serum samples were aliquoted and stored frozen at −80°C until further analysis. Afterwards, serum CRP levels were measured at the Central Facility for Clinical Chemistry of the University Hospital Ulm using a chemiluminescence immunoassay analyzed on a Cobas 6,000 platform (Roche Diagnostics, Risch, Switzerland) for study cohort I and on a Cobas 8,000 platform (Roche Diagnostics, Risch, Switzerland) for study cohort II. One participant of study cohort I and three participants of study cohort II showed a CRP level >10 mg/l, which is indicative of an acute infection, and were therefore excluded from all subsequent analyses.

Measures in Study Cohort I

Study Participants of Study Cohort I

In study I (conducted from March 2012–May 2013), a total of 240 women gave written informed consent and participated in the screening interview (t_0). Oversampling for individuals with a higher maltreatment load, 112 women were invited and 67 actually participated at the follow-up interview 3 months postpartum (t_1; see **Supplementary Figure S1** for detailed description of study flow and drop-out rates). Applying the established cut-off criteria of the CTQ (44, 45), 25 of these women were categorized as having no CM experiences, 22 as having low CM experiences, five as having moderate CM experiences, and 15 as having severe CM experiences. As study participants with moderate and severe CM experiences were significantly younger than women with no or low CM experiences [$F_{(3,47)} = 3.76$, $p = 0.017$], a subsample of 31 participants was selected out of this total study cohort to match women with no and low CM experiences and women with moderate and severe CM experiences for age [see (34) for a detailed description]. Body mass index (BMI) was a secondary matching criterion as BMI influences levels of oxidative stress (48, 49).

The final study cohort selected for biological analyses consisted of $N = 8$ women with no, $N = 8$ women with low, $N = 4$ women with moderate and $N = 11$ women with severe CM experiences. Additionally, one study participant with low CM experiences was subsequently excluded from the analyses, as the serum CRP level indicated the presence of an acute inflammatory status [see Serum C-reactive protein (CRP) content]. Thus, reported statistical data of study cohort I are based on a final sample of $N = 30$ women (see **Supplementary Figure S2** for the distribution of the maltreatment load).

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

For the assessment of nuclear DNA damage, PBMC were isolated from whole blood by Ficoll-Hypaque gradient centrifugation according to the manufacturer's protocol (GE Healthcare, Chalfont St. Giles, UK) immediately after blood sampling. Isolated cells were stored at −80°C in cryopreservation medium (dimethyl sulphoxide: Sigma-Aldrich, St. Louis, MO, USA; fetal calf serum: Sigma-Aldrich; dilution 1:10). For the analyses, frozen PBMC were thawed, washed twice in phosphate-buffered saline (PBS) at room temperature and counted with trypan blue staining for the quantification of living cells. An aliquot of 1×10^5 cells was then used for the detection of nuclear strand breaks by comet assay and an aliquot of 5×10^5 cells was fixated in a 3:1 (v/v) solution of methanol (Sigma-Aldrich) and glacial acetic acid (VWR, Radnor, PA VWR, Radnor, PA, USA) for the detection of γH2AX foci.

Comet Assay

The comet assay measures DNA strand breaks (single strand and double strand breaks) after lysis of the cells (9). The alkaline version of the comet assay (single-cell gel electrophoresis) was performed on PBMC as previously described by Speit and Hartmann (50). In short, 5×10^4 cells were suspended in an agarose gel on a microscopy slide. Following lysis (for at least 1 h), cells were denatured with alkali (pH 13) for 30 min and electrophoresis was performed for 25 min at 25 V and 300 mA using a Consort Electronics power supply ev231 (CONSORT, Turnhout, Belgium). Slides were subsequently stained with ethidium bromide (Roth, Karlsruhe, Germany) for the analysis of the DNA migration distance by fluorescence microscopy (Olympus BX41 U-LH100HG, Olympus, Tokyo, Japan; **Supplementary Figure S3**). The software Comet Assay II (Perceptive Instruments, Haverhill, UK) was used to determine the median tail intensity (percentage of DNA in the tail) and median tail moment (tail intensity × tail length) of 100 randomly selected cells per slide on two slides per sample. For each run, a positive control (x-ray irradiated Hela cells) and a negative control (non-irradiated Hela cells) were included. The measures tail intensity and tail moment were used for statistical analyses.

Detection of γH2AX Foci

As a marker for DNA double strand breaks (10), we measured phosphorylated histone H2AX (γH2AX) in intact cells. For fluorescence staining, 1×10^5 fixated cells were spread out onto superfrost slides (Menzel-Glaeser, Braunschweig, Germany) and washed with PBS (2 × 5 min). Subsequently, cells were permeabilized with pepsin for 10 min at 37°C, washed twice in washing buffer (70% [v/v] formamide, 10 mM Tris base, 0.1% [w/v] bovine serum albumin) for 20 min each, twice in TBS-Tween (1%) for 5 min each, and twice in PBS for 5 min each. All cover slips were then treated with 200 µl primary antibody solution (Anti-phospho-Histone H2A.X [Ser139], Merck, Millipore, Billerica, MA, USA) diluted 1:1,000 in blocking buffer (0.9 M PBS, 19% [w/v] bovine serum albumin, 0.1% v/v Tween 20) and incubated over night at 4°C. On the next day, the slides were washed with PBS (2 × 5 min) and then incubated with 200 µl secondary antibody solution (goat anti-mouse Alexa-Fluor 488 nm, Life Technologies, Carlsbad, CA, USA; dilution 1:300 in blocking buffer) for 1 h in a humid chamber at room temperature. Thereafter, the slides were washed with PBS (2 × 5 min). Finally, cell nuclei were counterstained with DAPI using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Analysis of γH2AX was performed using a Leica DM5000 B fluorescent microscope (Leica Microsystems, Wetzlar, Germany), and images were taken at a 1,000-fold magnification and an exposure time of 400 ms (**Supplementary Figure S4**). One hundred cells per sample were assessed and the number of γH2AX foci was counted manually. In each run, a positive control (x-ray irradiated Hela cells) and a negative control (non-irradiated Hela cells) were included. For statistical analyses, the following two measures were applied: the number of γH2AX foci per cell (γH2AX foci/cell) and the percentage of cells with γH2AX foci.

Analyses in Study Cohort II

Study Participants of Study Cohort II

The participants of study cohort II were recruited within the "My Childhood—Your Childhood" project which was conducted from October 2013 to December 2016. After providing written informed consent, 533 women participated at t_0 in study II [see (51) for a detailed description]. Three months postpartum, 285 of these women participated at t_1 (see **Supplementary Figure S5** for detailed description of study flow and drop-out rates) in a detailed psychodiagnostic interview. Lifetime psychiatric disorders were diagnosed by trained psychologists with the German version of the *Structured Clinical Interview* (SCID-I) (52) for the diagnosis of major axis I disorders of the *Diagnostic and Statistical Manual of Mental Disorders* [4th ed., text rev.; DSM-IV-TR; (53)].

At t_1, we were able to obtain peripheral blood samples from 252 women for the generation of serum and plasma samples. According to the established cut-off criteria of the CTQ (44, 45), 141 of these women were categorized as having no CM experiences, 52 as having low CM experiences, 28 as having moderate CM experiences, and 31 as having severe CM experiences. In order to validate the association between the maltreatment load and oxidative stress biomarkers in a sample that was controlled for potential confounding factors known to influence oxidative stress biomarkers such as current cigarette smoking (54) and BMI (48, 49), we excluded women who reported current smoking at t_1 ($N = 14$: $N_{\text{no CM}} = 6$, $N_{\text{low CM}} = 3$, $N_{\text{moderate CM}} = 2$, and $N_{\text{severe CM}} = 3$) and women

with a BMI > 30 kg/m^2; $N = 22$: $N_{\text{no CM}} = 10$, $N_{\text{low CM}} = 3$, $N_{\text{moderate CM}} = 4$, and $N_{\text{severe CM}} = 5$) from the biological analyses in study cohort II. Furthermore, women with autoimmune diseases ($N = 12$: $N_{\text{no CM}} = 7$, $N_{\text{low CM}} = 2$, $N_{\text{moderate CM}} = 2$, and $N_{\text{severe CM}} = 1$), non-Caucasian ethnicity ($N_{\text{no CM}} = 1$), acute intake of psychotropic medication ($N_{\text{severe CM}} = 1$), acute illness (self-report; $N = 24$: $N_{\text{no CM}} = 14$, $N_{\text{low CM}} = 5$, $N_{\text{moderate CM}} = 3$, and $N_{\text{severe CM}} = 2$), and missing psychological data ($N_{\text{low CM}} = 1$) were excluded. For women without CM experiences, a lifetime history of a psychiatric disorder ($N = 28$) and experiences of severe distress within the last 3 months (e.g., death of a close person; $N = 10$) were applied as further exclusion criteria. From the remaining $N = 65$ women without CM experiences, $N = 46$ were selected for oxidative stress analysis due to limited capacity of financial resources. Additionally, three study participants (two women with no CM experiences and one with severe CM experiences) were excluded from the statistical analyses as the serum CRP levels indicated the presence of an acute inflammatory status [see Serum C-reactive protein (CRP) content]. To this end, the final study cohort II ($N = 117$) consisted of 44 women with no CM experiences, 38 women with low CM experiences, 17 women with moderate CM experiences and 18 women with severe CM experiences. CM experiences (see **Supplementary Figure S6** for the distribution of the maltreatment load).

Blood Sampling

In study cohort II, oxidative stress parameters were assessed in serum and plasma samples. Therefore, whole blood (one pre-chilled S-Monovette for serum and one pre-chilled EDTA-Monovette for plasma sampling), was centrifuged for 10 min at 3,000 g and 4°C. Serum and plasma samples were aliquoted and stored frozen at −80°C until further analysis. Serum samples were used for the quantification of 8-OH(d)G and plasma samples for the assessment of free and total 8-isoprostane levels.

Oxidative Stress Parameters in Serum and Plasma

Serum 8-OH(d)G levels were quantified using the *DNA/RNA Oxidative Damage ELISA Kit* (Item No. 589320, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. This immunoassay covers three oxidized guanine species as marker for DNA/RNA oxidative damage: 8-hydroxy-2′-deoxaguanosine from DNA, 8-hydroxyguanosine from RNA and 8-hydroxyguanine from either DNA or RNA. As recommended in the manufacturer's protocol, serum samples were diluted 1:25 prior to analysis. The assay has a range from 10.3 to 3,000 pg/ml and a sensitivity of approximately 30 pg/ml. As markers for lipid peroxidation, free (circulating) and total 8-isoprostane levels were measured in blood plasma. Total 8-isoprostane is a combination of free 8-isoprostane and 8-isoprostane that is esterified to phospholipids. Free and total plasma 8-isoprostane levels were measured using the *8-isoprostane ELISA Kit* (Item No. 516351, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. The assay has a range from 0.8 to 500 pg/ml and a sensitivity of approximately 3 pg/ml. For the measurement of free 8-isoprostane, plasma samples were used untreated, whereas an additional alkaline hydrolysis step was performed for the analysis of total 8-isoprostane. Analyses were performed in thawed samples in duplicates and averaged values were used for statistical analyses. Samples were randomly distributed over the plates to prevent any batch effects.

Statistical Analyses

All biological analyses were performed blinded with respect to clinical variables. Statistical analyses were performed using R version 3.5.0 (55) and p-values <0.05 were considered as significant. In accordance with the findings that the risk for developing PTSD after traumatic experiences increases with increasing traumatic load (56, 57), there is accumulating evidence pointing toward a dose-response-relationship between the severity of maltreatment experiences (maltreatment load) and associated biological alterations (34, 51, 58, 59). Therefore, we tested for an association between cellular, serum, and plasma biomarkers of oxidative stress-related damages and the CTQ sum score as a continuous measure for the maltreatment load. Due to skewness, non-normality, and outliers in oxidative stress measures as well as in the maltreatment load, the use of traditional parametric methods was inappropriate. Thus, the nonparametric probabilistic index model (PIM) of Thas et al. (60), a robust rank-based equivalent of the generalized linear model, was applied [R package "pim" version 2.0.0.2: (61)]. Due to the relatively small sample size, no covariates were included in the statistical analyses in study cohort I, which was, however, matched for age and BMI to minimize the influence of these potential confounders. For study cohort II, the influence of potential confounders for oxidative stress measurements (smoking, obesity, autoimmune diseases, non-Caucasian ethnicity, acute intake of psychotropic medication, and acute illness) was minimized using exclusion criteria (see Study participants of study cohort II). Age was included as covariate in the statistical analyses of study cohort II as oxidative stress was found to be involved in aging (62). The probability (P) for an increase of the outcome variable was modeled as a function of the predictors (study cohort I: maltreatment load; study cohort II: maltreatment load and age). The estimates (b), 95% confidence intervals (CI) of the estimates (b[95% CI]), standard errors (SE b), as well as related z-statistics and p-values were used for these rank-based regression models.

RESULTS: CHILDHOOD MALTREATMENT AND OXIDATIVE STRESS BIOMARKERS IN STUDY COHORT I AND STUDY COHORT II

All descriptive sociodemographic and biological data of study cohort I and study cohort II can be found in **Tables 1** and **2**, respectively. The levels of humoral oxidative stress markers (8-OH(d)G, free 8-isoprostane, and total 8-isoprostane) in maternal blood at t_1 did not differ significantly between the different types of delivery at t_0 (all $p > 0.18$). For a graphical overview of the biological raw data see **Supplementary Figure S7**. All results

TABLE 1 | Socio-demographic and clinical data of study cohort I and study cohort II.

	Study cohort I (*N* = 30)		Study cohort II (*N* = 117)	
	Mean ± SD or *N* (%)	**Range**	**Mean ± SD or *N* (%)**	**Range**
DEMOGRAPHICS				
Age (years)	31.6 ± 6.0	22 – 44	33.0 ± 4.1	23 – 43
BMI (kg/m^2)	25.3 ± 6.4	19 – 47	23.7 ± 3.0[a]	16.5 – 30
Smoking status [yes, *N* (%)]	8 (28.6 %)[b]	–	–	–
Ethnicity [Caucasian, *N* (%)]	29 (96.7 %)[c]	–	117 (100 %)	–
Number of children	2 ± 1	1 – 5	2 ± 1[a]	1 – 4
Living in a partnership [yes, *N* (%)]	30 (100 %)		114 (99.1 %)[d]	–
Academic education [yes, *N* (%)]	13 (43.3 %)	–	88 (75.9 %)[a]	–
Vaginal delivery [yes, *N* (%)]	30 (100 %)	–	89 (76.7 %)[a]	–
Time interval between last food intake and blood drawing (minutes)	125.5 ± 125.9[e]	5 – 480	114.7 ± 76.1[a]	0 – 333
ADVERSITY AND PSYCHIATRIC SYMPTOM LOAD				
CTQ sum score	42.8 ± 14.2	25 – 73	35.0 ± 10.0	25 – 81
Emotional abuse sum score	9.8 ± 5.3	5 – 21	7.4 ± 3.4	5 – 21
Physical abuse sum score	7.1 ± 3.8	5 – 18	6.0 ± 2.5	5 – 21
Sexual abuse sum score	6.7 ± 4.1	5 – 25	5.9 ± 3.1	5 – 21
Emotional neglect sum score	12.3 ± 4.8	5 – 22	9.9 ± 3.9	5 – 18
Physical neglect sum score	6.9 ± 2.9	5 – 16	5.9 ± 1.8	5 – 15
Psychiatric diagnoses lifetime	**Self-report**		**SCID diagnosis**[a]	
Depressive disorder [*N* (%)]	6 (20.0 %)	–	15 (12.9 %)	–
Anxiety disorder [*N* (%)]	2 (6.7 %)	–	21 (18.1 %)[f]	–
Borderline personality disorder [*N* (%)]	2 (6.7 %)[g]	–	–	–
Eating disorder [*N* (%)]	1 (3.3 %)[h]	–	–	–
Compulsive disorder [*N* (%)]	1 (3.3 %)	–	1 (0.9 %)	–
Alcohol use disorder [*N* (%)]	–	–	4 (3.4 %)	–
Stimulant use disorder [*N* (%)]	–	–	2 (1.7 %)	–
Trauma–related disorders [*N* (%)]	–	–	4 (3.4 %)	–
CHRONIC ILLNESSES[i]	**Self-report**		**Self-report** [a]	
Thyroid disease [*N* (%)]	5 (16.7 %)	–	22 (19.0 %)	–
Hypertension [*N* (%)]	2 (6.7 %)	–	–	–
Allergy [*N* (%)]	1 (3.3 %)	–	2 (1.7 %)	–
Asthma [*N* (%)]			7 (6.0 %)	–
Epilepsy [*N* (%)]	–	–	2 (1.7 %)	–
Neurodermatitis [*N* (%)]	–	–	2 (1.7 %)	–
MEDICATION[j]	**Self-report**		**Self-report**[a]	
L-Thyroxin [*N* (%)]	4 (13.3 %)	–	26 (22.4 %)	–
Psychotropic medication [*N* (%)]	3 (10.0 %)	–	–	–
Oral contraceptives [*N* (%)]	1 (3.3 %)	–	14 (12.1 %)	–
Analgesic [*N* (%)]	–	–	6 (5.2 %)	–
Asthma inhaler [*N* (%)]	–	–	2 (1.7 %)	–

BMI, Body mass index; CTQ, Childhood Trauma Questionnaire; SCID, Structured Clinical Interview.
[a]N = 116, one missing value. [b]N = 28, two missing values. [c]One study participant of Brazilian origin. [d]N = 115, two missing values. [e]N = 27, three missing values. [f]One subject with depressive disorder, anxiety disorder and trauma-related disorder. [g]One subject with lifetime Borderline personality disorder and anxiety disorder. [h]One subject with lifetime diagnosis of eating disorder and mild depression. [i]Chronic illnesses that have been reported by more than two study participants. In study cohort I, one study participant reported each the following chronic illnesses: asthma, chronic bronchitis, colitis ulcerosa, epilepsy, and psoriasis vulgaris. In study cohort II, one study participant reported each the following chronic illnesses: chronic venous insufficiency, circular hair loss, coagulation disorder, hay fever, prediabetes, prolaktinoma, protein S deficiency, prothrombin mutation, Scheuermann's disease, scoliosis, and von Willebrand disease. [j]Medication reported if at least 2 study participants reported intake.

of the probabilistic index models reported in the following are summarized in **Table 3**. With regard to cellular measures of (oxidative) DNA damage assessed in study cohort I, the analyses revealed no significant main effects of maltreatment load—as measured by the CTQ sum score—on tail intensity ($b = -0.0011$, $p = 0.95$) and tail moment ($b = -0.0035$, $p = 0.83$). These results were confirmed by γH2AX fluorescence staining, with no significant main effects of maltreatment load on γH2AX foci/cell ($b = 0.0065$, $p = 0.68$) and the percentage of cells with γH2AX foci ($b = -0.0004$, $p = 0.97$).

TABLE 2 | Cellular, serum, and plasma measures of oxidative stress biomarkers in study cohort I and study cohort II.

	Mean ± SD	Range	Median	IQR
STUDY COHORT I (*N* = 30)				
Tail Intensity (%)	2.97 ± 1.64	0.74–8.52	2.60	1.75
Tail Moment (AU)	0.26 ± 0.16	0.08–0.90	0.24	0.16
γH2AX foci/cell[a]	0.31 ± 0.28	0.01–1.03	0.22	0.30
% cells with γH2AX foci[a]	18.97 ± 14.23	1–50	16	20
STUDY COHORT II (*N* = 117)				
8-OH(d)G levels (pg/ml)	4456 ± 1076	1336–7480	4343	1571
Free 8-isoprostane levels (pg/ml)	36.8 ± 79.5	2.1–538.2	20.1	11.9
Total 8-isoprostane levels (pg/ml)	364.7 ± 187.5	97.9–1094.2	323.0	254.5

[a]*N = 29.*

TABLE 3 | Probabilistic Index Model results on the association between the CTQ sum score and cellular (study cohort I), serum, and plasma measures (study cohort II) of oxidative stress-related damage.

Regressor	b	b [95% CI]	SE	z	*p*
STUDY COHORT I (*N* = 30)					
Tail Intensity	−0.0011	[−0.0333;0.0312]	0.02	−0.06	0.95
Tail Moment	−0.0035	[−0.0348;0.0279]	0.02	−0.22	0.83
γH2AX foci/cell[a]	0.0065	[−0.0249;0.0380]	0.02	0.41	0.68
% cells with γH2AX foci[a]	−0.0004	[−0.0298;0.0288]	0.02	−0.03	0.97
STUDY COHORT II (*N* = 117)[b]					
8-OH(d)G	0.0155	[−0.0076;0.0386]	0.01	1.32	0.19
Free 8-isoprostane	0.0277	[0.0065;0.0490]	0.01	2.56	0.01
Total 8-isoprostane	0.0187	[−0.0043;0.0417]	0.01	1.60	0.11

CI, Confidence interval; CTQ, Childhood Trauma Questionnaire.
[a]*N = 29.*
[b]*Probabilistic Index Models in study cohort II included maternal age as covariate.*

The analyses of serum and plasma oxidative stress biomarkers assessed in study cohort II revealed a significant main effect of maltreatment load on free 8-isoprostane levels ($b = 0.0277$, $p = 0.01$), but not on total 8-isprostane ($b = 0.0187$, $p = 0.11$) and 8-OH(d)G ($b = 0.0155$, $p = 0.19$) levels. Thus, the probability for higher free 8-isoprostane levels increased significantly with a higher maltreatment load (**Figure 1**). No significant main effects were found for the covariate age on free and total 8-isoprostane and 8-OH(d)G levels, respectively ($p > 0.05$). Results remained the same when one outlier in total 8-isoprostane (1094.2 pg/ml) and four outliers in free 8-isoprostane (>200 pg/ml) were excluded from the respective analyses. Including the time interval between the last food intake and blood drawing as additional covariate in our statistical analyses of study cohort I and study cohort II did not alter the significance of the results.

DISCUSSION

We comprehensively assessed serum, plasma, and cellular measures that are well-established biomarkers of oxidative stress-related damages—i.e., DNA/RNA damages and lipid peroxidation—over two study cohorts of postpartum women with a history of childhood maltreatment. In both cohorts, we previously found indications for oxidative imbalances in relation to CM: in study cohort I we showed that CM was associated with increased ROS production and reduced levels of L-carnitine and acetylcarnitine, two serum metabolites that inherit antioxidant capacities (34), while untargeted metabolomics analyses revealed a higher signal intensity for bilirubin in CM-affected individuals of study cohort II (35). Bilirubin is an end product of heme degradation by HO-1. The enzyme itself as well as its degradation products, like bilirubin, were reported to have immunomodulatory, anti-inflammatory, and anti-oxidative properties (36–38). Building on these results, the analyses of two cellular measures of nuclear DNA damage (DNA migration in the comet assay and the appearance of γH2AX foci within the cell nucleus) indicated now, however, that CM and in particular the maltreatment load was not related to an increase in oxidative DNA damage in PBMC in study cohort I. This finding was further supported by the analyses conducted in study cohort II, a cohort controlled for the potential confounding factors smoking and BMI, where we did neither find a significant association between maltreatment load and the serum levels of 8-OH(d)G. Thus, by combining three different methods and two study cohorts, we consistently found that CM was not related to oxidative DNA damages in postpartum women. With regard to lipid peroxidation, we found that an increasing maltreatment load was significantly associated with a higher probability for increased plasma levels of free 8-isoprostane, but not with plasma levels of total 8-isoprostane in study cohort II. In line with previous findings from our group (34, 51, 58, 59), we found with regard to free 8-isoprostane level again a significant influence of the severity of CM experiences, thus supporting the hypothesis of a dose-dependent effect of maltreatment load. Together, these results bear several potential suggestions: (1) that an increase in ROS levels and associated oxidation products in CM-affected individuals might not only be seen with regard to its damaging potential, but might instead serve a functional role, (2) that CM-related oxidative damages may be persistent at the level of lipid peroxidation, while DNA repair mechanisms may counterbalance and thus cope with oxidative stress-induced DNA damages, and (3) that exogenous or endogenous resilience factors may influence the association of CM with oxidative stress-related damages. These potential implications will be discussed in the following.

Consistent with our findings, two previous studies did not report any significant associations between childhood abuse and neglect and serum 8-OH(d)G levels in children (33) and in adults with and without different personality disorders (23). Fanning et al. (23) further found in the same cohort no association between CM and plasma 8-isoprostane levels, but the authors did not specify whether they assessed free or total 8-isoprostane. While we found no significant alterations in total 8-isoprostane levels, we found a significantly increased probability of higher free 8-isoprostane plasma levels with higher maltreatment load in postpartum women. In contrast to esterified 8-isoprostane that constitutes the major fraction of total 8-isoprostane levels, free 8-isoprostane can not only be generated by the non-enzymatic, ROS-mediated peroxidation of

arachidonic acid, but also by an alternate enzymatic pathway that is catalyzed by the inflammation-induced *prostaglandin-endoperoxidase synthase* (63–66). It was therefore recently suggested that an increase in total 8-isoprostane may be indicative of oxidative stress, whereas a sole increase in free 8-isoprostane may rather point to the involvement of inflammatory processes (15). For the interpretation of 8-isoprostane results, it is therefore necessary to always consider both pathways, the oxidative stress-related and the inflammation-related pathway. With regard to these two pathways, Eick et al. (30) previously reported a higher chemical fraction of 8-isoprostane urine levels in pregnant women with poor psychosocial status (e.g., high anxiety levels, high depression levels, low self-esteem, low mastery, and high subjective stress), but no difference in the enzymatic fraction by investigating the ratio of 8-isoprostane to prostaglandin F2α. These results point toward the presence of increased oxidative stress levels in association with psychosocial disadvantages as well as with extremely stressful life events, such as family death, during pregnancy (30). In contrast, we found a significant association between the severity of maltreatment experiences in childhood and free 8-isoprostane levels, but not total 8-isoprostane levels. Thus, our findings in study cohort II might be indicative of increased chronic inflammatory processes associated with CM in postpartum women rather than of increased oxidative stress. CM has been consistently associated with a phenotype of chronic low-grade inflammation [reviewed in (67)]. In line with this suggestion, we previously found in study cohort I not only that CM was associated with increased immuno-cellular ROS production in postpartum women, but also that this increase in ROS production was further associated with a pro-inflammatory status of the cells (34). Indeed, mounting evidence indicates that ROS are not only by-products of mitochondrial oxidative phosphorylation, but also have important signaling functions and are involved in pathways regulating anti-microbial effects (68), apoptosis (69), autophagy (70), and inflammation (39, 40). Excessive ROS production by mitochondria can drive the gene-expression and production of pro-inflammatory cytokines through activation of pro-inflammatory transcription factors (e.g., NFκB) and through activation of the NLRP3 inflammasome (39, 40). Subsequently, inflammation can also induce ROS production by inflammatory cells leading to higher levels of oxidative stress (5). Thus, the observation of increased ROS production and ROS-related oxidation products with CM could be a sign of inflammatory signaling processes rather than for high oxidative stress levels causing cellular damage.

A second potential explanation for the observed difference in oxidative DNA/RNA and lipid damages with CM might lie in a difference in repair, metabolism, and excretion dynamics. In contrast to lipids, DNA repair mechanism may counterbalance and thus cope with oxidative stress-induced DNA damages (71, 72). As such, lipid peroxidation might persist and constitute a long-term marker of stress experiences, while oxidative DNA damages might rather be observable in association with acute stress experiences or it might take more severe levels of psychological and oxidative stress to induce persistent oxidative DNA damages. Consistent with this hypothesis, it was recently reported that adult psychiatric patients with a history of complex childhood traumatization presented significantly higher levels of nuclear DNA damage in PBMC as assessed by γH2AX staining compared to healthy individuals and also compared to psychiatric patients with low levels of complex childhood traumatization (32). Complex childhood traumatization was here defined as the experience of sexual, physical or emotional abuse by a primary caregiver or another member of the family or social group the victim belongs to (32). Investigating refugees with a high traumatic load, we also reported that individuals with PTSD showed higher levels of basal DNA breakage in PBMC compared to trauma-exposed subjects without PTSD and non-trauma-exposed control subjects (21). Individuals with PTSD showed, however, a higher cellular capacity to repair single-strand breaks after exposure to ionizing x-radiation (21), which may point toward a trauma-specific effect on cellular DNA repair processes. Cellular repair mechanisms of oxidative DNA damages may play an even stronger role in protecting the DNA against mutations, which can—if they are not recognized and repaired—lead to a higher risk for somatic diseases like cancer (73).

Although CM constitutes a major risk factor for both adverse mental and physical disorders, not all individuals with a history of CM develop pathological health outcomes in the long-term. It therefore has been suggested that individual vulnerability and resilience factors such as the genetic background, but

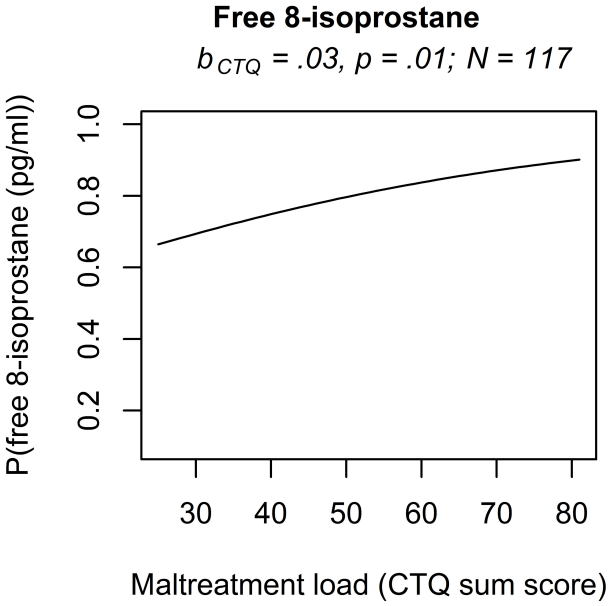

FIGURE 1 | Effect for the maltreatment load (CTQ sum score) on free 8-isoprostane in study cohort II. The results of the rank-based regression model for free 8-isoprostane are visualized as the probability (P) for an increase of free 8-isoprostane modeled as a function of the predictors (maltreatment load (CTQ sum score) and age as covariate). Free 8-isoprostane was measured as marker for lipid peroxidation in study cohort II ($N = 117$). b_{CTQ}, Estimate for the main effect of the CTQ sum score; CTQ, *Childhood Trauma Questionnaire*; P, Probability for an increase of the outcome variable modeled as a function of the predictors.

also environmental, behavioral, and psychosocial factors can influence "how deep CM gets under the skin" (74, 75). In line with this suggestion, our working group showed that women with CM reported lower levels of perceived stress, if they concomitantly reported higher levels of social support (76). We further showed that the stress-related hormone cortisol potentiates the effect of CM on telomere length shortening and on the increase in immune-cellular oxygen consumption (42, 59). On the other hand, the attachment-related hormone oxytocin may buffer the biological effects of childhood maltreatment on telomere length and cellular oxygen consumption (42, 59). Furthermore, there is first evidence that nutrition like the supplementation with omega-3 fatty acid has beneficial effects on lipid peroxidation (77). Future studies should therefore take genetic, psychosocial, behavioral, and biological factors into account to further dissect the association of CM with oxidative stress states and related cellular and structural damages.

While the present study has several strengths such as the consistency of the observed findings across different research methods across two study cohorts, there are also some limitations: Biological assessment in our study was conducted three months postpartum. The postpartum period is characterized by major life transitions, which are particularly stressful for mothers with a history of CM (76). Therefore, increased current perceived stress and adverse childhood experiences are comorbid and it is difficult to disentangle the effects on oxidative stress markers. By investigating this specific study cohort, we were able to analyze differences in oxidative stress markers with respect to negative childhood maltreatment experiences in a highly demanding and sensitive time period.

Pregnancy and parturition are not only associated with social and psychological alterations, but also with biological alterations characterized by substantial changes in the maternal immune and endocrine system (78, 79). Pregnancy and especially the third trimester is furthermore associated with increased oxidative stress markers in women with uncomplicated pregnancies (80). However, it was further reported that most of the oxidative stress markers had returned to non-pregnant levels 6 to 8 weeks postpartum and were comparable to those of non-pregnant and non-postpartum women (80). Furthermore, our reported oxidative stress values are comparable to those of non-pregnant women (15, 81). According to these findings, it can be assumed that pregnancy-related changes in the oxidative stress system had mostly normalized at the time point of biological assessment in our studies. Nevertheless, the results need to be replicated in non-postpartum women and investigated also in men to show the generalizability of the findings.

Our study cohorts consisted of healthy, non-clinical community samples with a relatively high socioeconomic status. As the socioeconomic status is a protective factor for mental health (82), the high socioeconomic status of our study cohorts might contribute to the observation of small effects of the maltreatment load on oxidative stress markers in blood.

Due to ethical considerations, we collected non-fasting blood samples for mothers who were potentially still breastfeeding their children three months postpartum. Non-fasting blood collection could also have an effect on the oxidative stress markers measured in blood, which should be analyzed in future studies. We comprehensively assessed serum, plasma, and cellular measures that are well-established and stable biomarkers of oxidative stress-related damages. However, they are all indirect markers for oxidative stress. Future studies should use new technologies, for example electro-spin-resonance (ESR), to directly measure ROS in blood and biological fluids (83).

Furthermore, the intake of medication, mainly of thyroid hormones, as well as the presence of comorbid diseases, both somatic and psychiatric disorders, might have an effect on the measured oxidative stress levels. Exclusion of individuals with somatic or psychiatric disorders would lead to a non-representative study cohort as negative health outcomes are observed at higher rates in CM-affected individuals (1). Nevertheless, the influence of different co-morbid chronic and psychiatric disorders in individuals with a history of CM on oxidative stress parameters has to be investigated in more detail in further studies.

CONCLUSION

In conclusion, a history of CM was associated with higher plasma levels of free 8-isoprostane, but not with total 8-isoprostane in postpartum women. By combining different methods and two study cohorts, we found no indications for higher oxidative DNA damages with higher maltreatment load in postpartum women. Further research is needed to investigate whether the increase in free 8-isoprostane is a persistent marker for oxidative stress or whether it is instead functionally involved in ROS-related signaling pathways that potentially regulate inflammatory processes following a history of CM. Additionally, even in non-psychiatric cohorts with CM, possible treatment effects by behavioral, psychotherapeutic, or mental stress coping interventions should be investigated for their protective potential against the biological sequelae of early life adversities to reduce the risk for mental as well as physical health conditions in the aftermath of CM.

AUTHOR CONTRIBUTIONS

Study cohort I was part of a pilot project to show the feasibility of study II. Study cohort II was part of the project "My Childhood—Your Childhood," funded by the Federal Ministry of Education and Research of Germany between 2013 and 2016. Both projects were, among others, conceptualized by I-TK, and AKa. For both studies, I-TK provided additional funding for the biological analyses and supervised all stages of the project. AKo recruited the women, performed the screening as well as diagnostic interviews, collected and preprocessed clinical data. CB and AG conducted the biological analyses. PR contributed analytical tools. CB and AKo performed statistical analyses and interpreted the data together with AG, AKa, and I-TK. AG and CB wrote the manuscript with input and critical revisions from all authors. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00023/full#supplementary-material

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MINI REVIEW
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Stressful Newborn Memories: Pre-Conceptual, *In Utero*, and Postnatal Events

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Early-life stressful experiences are critical for plasticity and development, shaping adult neuroendocrine response and future health. Stress response is mediated by the autonomous nervous system and the hypothalamic–pituitary–adrenal (HPA) axis while various environmental stimuli are encoded *via* epigenetic marks. The stress response system maintains homeostasis by regulating adaptation to the environmental changes. Pre-conceptual and *in utero* stressors form the fetal epigenetic profile together with the individual genetic profile, providing the background for individual stress response, vulnerability, or resilience. Postnatal and adult stressful experiences may act as the definitive switch. This review addresses the issue of how preconceptual *in utero* and postnatal events, together with individual differences, shape future stress responses. Putative markers of early-life adverse effects such as prematurity and low birth weight are emphasized, and the epigenetic, mitochondrial, and genomic architecture regulation of such events are discussed.

Keywords: stress, predisposition, epigenetics, low birth weight, individuality, early-life stress, mitochondria

STRESS, BRAIN, AND THE ENVIRONMENT

Physiological or biological stress is the response to a stressor, i.e., an environmental condition or a stimulus. The body responds to stress by sympathetic nervous system activation as a result of the fight-or-flight response. The stress response aims to restore homeostatic control and facilitate adaptation. The brain processes stress in three main areas: amygdala, hippocampus, and prefrontal cortex (PFC). Amygdala and hippocampus play a critical role in memory formation and are associated with anxiety, fear, and cognitive processes. PFC is the brain region linked to planning complex cognitive behavior, personality expression, decision making, and moderating social behavior (1). The basic activity of the PFC region is to orchestrate thoughts and actions in accordance with internal goals and executive function (2). Corticosteroid receptors that react to the stressor through steroid

Abbreviations: GR, glucocorticoid receptor; GRE, glucocorticoid response elements; HPA, hypothalamic–pituitary–adrenal; LBW, low birth weight; MR, mineralocorticoid receptor; PGGR, Primary Generalized Glucocorticoid Resistance (Chrousos syndrome); SNP, single-nucleotide polymorphism.

hormone binding are abundant in these areas (3, 4). It is well established that stressful experiences during critical periods of early brain development can affect emotional and behavioral functions in adult life (5). The autonomous nervous system and the hypothalamic–pituitary–adrenal (HPA) axis are responsible for these functions and mediate stress response through targeted hormone release. This system acts by negative feedback to maintain brain homeostasis. The hypothalamus is stimulated by its inputs and releases the corticotropin-releasing hormone. This hormone is transported to its target, the pituitary gland, where it binds to the targeted receptors and causes the release of the adrenocorticotropic hormone. Although the main purpose of this system is well understood, recent studies attempt to identify underlying genetic mechanisms of brain function modulating mediators of this system including adrenaline and neuropeptides (6). Glucocorticoids reach the brain through the peripheral blood flow, where they bind to specific types of cytoplasmic glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). MRs make up the majority of stress corticosteroid receptors with a high affinity for cortisol and are activated as soon as a stressor appears. GRs have a low affinity for cortisol and are only activated when stress reaches its peak on the brain. This complex is then translocated to the nucleus, where it binds to specific DNA elements [glucocorticoid response elements (GREs)] and acts as a transcription factor activating or repressing a great number of genes (7).

EARLY-LIFE STRESS, LEARNING, AND MEMORY

Exposure to early-life stressful events has been shown to activate the HPA stress hormone system. HPA axis mediator and receptor genes are prime targets of epigenetic modifications by DNA methylation and histone acetylation (8). The combination of genetic and epigenetic factors affects cell function and brain development. As a result, individuals who have experienced chronic stress during early development and childhood are at high risk for a wide range of behavioral problems that persist into adulthood. This phenotype becomes evident by learning and emotion regulation difficulties, alcohol and substance abuse, externalizing problems, as well as depression and anxiety disorders (7). Children who have experienced maltreatment or were exposed to maternal deprivation trauma have shown poor performance in tasks involving working memory, attention, planning, and learning processes (9, 10). In rodents, maternal deprivation is a well-established paradigm of early-life stress. Maternal deprivation of newborns from their dam leads to epigenetic changes in specific imprinted genes and dysfunctions. Behavioral and molecular effects depend on the duration and type of maternal deprivation and individual predisposition (11).

IN UTERO STRESS EXPOSURES

Intrauterine life events may have a much greater impact on epigenetic profiles than stressful exposures during adult life (12). Early stages of embryonic development are characterized by heightened brain plasticity that is adversely affected by exposure to environmental insults (13). Complex gene environment interactions during critical early developmental periods may have lasting effects and result in adult psychopathology (14, 15). Maternal stress exposure, anxiety, and depression during pregnancy are considered *in utero* adverse experiences and have been associated with low birth weight (LBW) and future health problems (16–24). LBW, apart from being a risk factor for neonatal morbidity and mortality, has been proposed as a marker of early-life adversities (25, 26).

In this mini-review, genetic and epigenetic factors that shape stress response are discussed. The contribution of mitochondria and individual predisposition to developing mental health problems in response to a stressful stimulus will also be addressed.

GENETICS AND EPIGENETICS OF THE STRESS RESPONSE

Early-life adversities have been implicated in the occurrence of neuropsychiatric conditions, such as, Post-traumatic stress disorder (PTSD), depression, psychosis, and phenotypes resembling mood- and anxiety-related disorders (4–8). Recent data are beginning to unravel the complex interactions between genes and environment, namely, an individual's genetic and epigenetic profile that renders the person resilient or at risk for developing a stress-related disorder (9, 10). Apart from the genetics of neuroendocrine stress response, it is important to take into consideration its epigenetic profile (11). A plethora of epigenetic marks, contributing to either the enhanced or suppressed expression of a gene, in combination with risk- or resilience-related predisposing polymorphisms, shape an individual's phenotype (27). The complex interaction of the genetic background with the epigenetic profile that reflects early-life experiences and is potentially reversible by environmental factors can result in a phenotype that is either resilient or sensitive towards adverse stress exposures (28, 29). Several genes and their epigenetic regulation have been implicated in the susceptibility to early-life stress. An overview of the below-discussed genes and their interrelations is provided in **Figure 1**.

NR3C1 and *NR3C2* genes (Nuclear Receptor Subfamily 3 Group C Member 1 and 2), encoding the GR and MR, respectively, are widely expressed in limbic regions of the brain and regulate HPA axis activity by cortisol binding. Deregulation of the GR–MR function may lead to HPA axis malfunction and stress vulnerability (43–45). The *NR3C1* gene, localized on the 5q31-32 chromosome, contains nine exons (1–9) (45, 46). In the 5′ Untranslated region (UTR), alternative splice variants of the first exon form the distal and proximal gene promoter that contains a crucial CpG island regulating the expression of exon 1_F. The multiple alternative first exon splice variants render the expression of *NR3C1* tissue-specific (47–50). The first study in humans examining the epigenetic status of 1_F promoter in low prenatal and increased maternal postnatal depression showed elevated methylation levels. This effect is reversed by maternal stroking of the newborns during the first postnatal weeks (51). In a thorough meta-analysis, psychosocial maternal prenatal stress

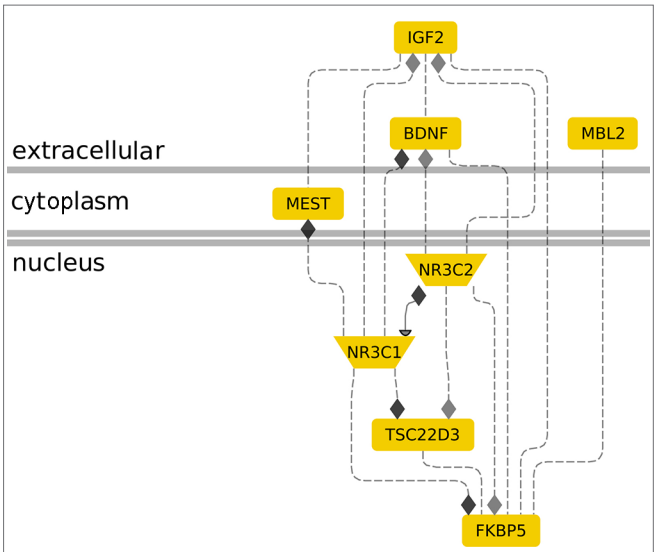

FIGURE 1 | The role of molecular genetic markers in cellular stress response. The interconnection between genes *IGF2, MBL2, MEST, NR3C1, NR3C2, TSC22D3*, and *BDNF* in the context of stress response is shown. Membrane-bound *IGF2* induction by maternal distress is associated with nuclear *NR3C2* induction (3). *IGF2* shows similar changes in methylation levels with NR3C1 during age-related stress (4), *FKBP5* during the development of preterm infants (30), and *MEST* in infertile males (31). *MBL2* and *FKBP5* upregulation has been associated with parental-nutrition-induced stress (32, 33). NR3C2 and *NR3C1* interact to control gene expression during stress (8, 34). Nuclear *NR3C1* (glucocorticoid receptor) seems to be a convergence point for *FKBP5* (9, 35), *NR3C2* (10, 36), *MEST* (11, 37), and *IGF2* (4) action in stress. *TSC22D3* is an established glucocorticoid signaling responsive gene that is regulated by *NR3C1* (27, 38) and an *NR3C2* target (28). *BDNF* is upregulated by *IGF2* (39) in an Alzheimer's disease mouse model, inhibited by *NR3C1* (40) in neuron-like cells, and associated with high *NR3C2* and low *NR3C1* in high-cholesterol-diet rats (41). *FKBP5* elevation is associated with *BDNF* suppression and improved anxiety, depression, and posttraumatic stress disorder conditions (42). Gene interaction analysis was performed using the Genomatix Pathway System (Genomatix.de).

was significantly correlated with DNA methylation at CpG 36 of the 1_F promoter (52). Interestingly, prenatal exposure of depressed mothers to serotonin reuptake inhibitors was not associated with alterations in the methylation profile of the 1_F promoter. However, a correlation between the psychological profiles of depressed mothers, especially during the third trimester, and increased HPA axis reactivity of the newborns, has been reported (53). Maternal anxiety during the first two trimesters also affects the methylation status of *NR3C1*, thus diminishing *NR3C1* gene expression (54). In a study examining the effects of maternal-related stressors such as maternal deprivation due to financial difficulties, daily psychosocial stress, and war-related phenomena, a strong correlation was found between the aforementioned maternal stressors, neonatal birth weight, and methylation of multiple CpG sites in the upstream *NR3C1* promoter. These results support the hypothesis that intrauterine development and maternal environmental stressors affect the plasticity and adaptation to adverse stimuli (55). Further supporting this notion, decreased expression of *NR3C1* was observed in hippocampal tissues of suicide completers abused during childhood. These findings can be explained by alterations in hippocampal methylation of tissue-specific *NR3C1*, which persist into adulthood and lead to changes in HPA axis function (56–58). The *NR3C2* gene on 4q31.1 has recently been associated with behavioral abnormalities. Cognitive ability following acute stress has been associated with genetic variation of the GR–MR. Specifically, single-nucleotide polymorphisms (SNPs) of the above genes seem to affect cognition and HPA axis function (59, 60). In individuals with a history of childhood maltreatment, the minor *NR3C2* allele rs17581262 was correlated, among others, with lower amygdala and hippocampal volumes and major depression, suggesting that this allele is a predisposing risk factor for stress-related disorders (61).

FKBP5 (6p21.31) encodes a 51-kDa immunophilin, which is a major component of the GR heterocomplex. Upon stress exposure, cortisol diffuses into the cytoplasm and binds the GR (62–64). *FKBP5* slows down the translocation of GR to the nucleus (65, 66). *FKBP5* expression is regulated by GREs *via* a cortisol-dependent short negative feedback loop (67, 68). In intron 2 of *FKBP5* and close to a functional GRE, the significant SNP rs1360780 was identified (69). Structurally, the rare risk allele alters the chromatin conformation after GR binding to the GRE, inducing the transcription of *FKBP5*. In the presence of the protective allele, this induction is absent (67, 69). The aforementioned SNP has been linked to a variety of mental health conditions including depression, anxiety, psychosis, and posttraumatic stress disorder (70–72). During their *in utero* formation, brain regions including the amygdala and hippocampus are particularly vulnerable in cases of antenatal maternal depression and anxiety (73, 74). *FKBP5* genetic variation among neonates combined with antenatal maternal depression can predispose toward the development of depressive symptoms in the offspring later in life due to alterations in neonatal brain regions (75). Interestingly, recent reports on the association of depression with childhood maltreatment did not report FKBP5 methylation to be involved in mediatory mechanisms (76, 77).

Alterations in GR function through *NR3C1* lead to a rare endocrinological condition known as Primary Generalized Glucocorticoid Resistance (PGGR, Chrousos syndrome) (78, 79). Mutations in the *NR3C1* gene result in receptor conformation changes and low ligand binding affinity and contribute to the clinical profile and pathogenesis (80–83). PGGR is characterized by decreased tissue sensitivity toward cortisol, resulting in malfunctioning negative feedback loops (84, 85). This causes a compensatory activation of the HPA axis and hypersecretion of its end products (80, 85, 86). Interestingly, *FKBP5* has been implicated in glucocorticoid resistance. The gene's overexpression is considered to be responsible for the low ligand-binding affinity of the GR in New World primates, providing a selective advantage of an overall normal adrenal function but with high concentrations of circulating Adrenocorticotropic hormone (ACTH) and cortisol (87, 88).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin expressed in hippocampus and PFC affecting neuron survival, development, and plasticity. Early-life stress and Val66Met polymorphism result in lower BDNF availability (29, 89).

The *GILZ* (glucocorticoid-induced leucine zipper) or *TSC22D3* gene, located on Xq22.2 (90), is induced by cortisol-bound GR. This complex binds on the GRE in the promoter of *GILZ*, thus

rendering this gene a valid measure of GR function (91–93). In an avian species, *GILZ* expression in the pituitary gland seems to be upregulated by glucocorticoids during the second half of the embryonic development and possibly plays a role in regulating pituitary hormone expression levels (94). *GILZ* is widely expressed in the brain, and its function depends on HPA axis activation. Increased expression of *GILZ* was found in the hippocampus and medial PFC of stressed mice, indicating a region-specific function (95). In human studies, decreased *GILZ* Messenger RNA (mRNA) levels were found in the PFC and the amygdaloid nuclei in teenage suicide completers (96). The above findings are only beginning to decipher the role of *GILZ* both in stress regulation and in immune system function.

GENETICS AND EPIGENETICS OF EARLY EMBRYONIC DEVELOPMENT

MBL2 (mannose binding lectin 2) is an important regulator of innate immunity and inflammatory processes. The *MBL2* gene encodes for a protein that assembles into a mannose-binding lectin complex. *MBL2* plays a very important role in the first-line immune responses, as a component of neonate immunity when the adaptive immunity system is not sufficiently developed (97). In humans, *MBL2* expression levels are determined genetically by a number of polymorphic sites of the gene as well as in its promoter region. Three non-synonymous SNPs, which are linked to absence or low levels of *MBL2*, have been identified in exon 1 and the promoter region. The most important *MBL2* gene SNPs associated with early infection and preterm delivery risk are variants B [rs1800450 (GGC→GAC)], C [rs1800451 (GGA→GAA)], and D [rs5030737 (CGT→TGT)]. Moreover, there are SNPs in the promoter region at position −550 in variant H/L (rs11003125) and at position −221 in variant X/Y (rs7096206) (25, 98). These *MBL2* gene polymorphisms are associated with an increased risk of perinatal and neonatal infections and risk of premature delivery (99, 100). *MBL2* levels could not predict the risk of newborn morbidity or mortality as a single factor since morbidity is also affected by other factors including sex, premature delivery, birth weight, etc. (97).

IGF2 (insulin growth factor 2), an imprinted gene, acts as a growth factor promoting differentiation and metabolism and plays an important role in the development and nutritional needs of the fetus (101). *IGF2* and *H19* are two genes of the same imprinted domain expressed from the paternal and maternal allele, respectively, that have been implicated in the control of placental and embryonic growth through cell proliferation and apoptosis (102, 103). *H19* is crucial for growth and differentiation of the placenta (104, 105).

MEST (mesoderm specific transcript, 7q32) is a paternally expressed imprinted gene, which influences placental and embryonic growth, as well as birth weight of the infant (31, 106). *MEST* is a member of the a/b-hydrolase superfamily and expressed in the embryonic mesoderm (107). Increased *MEST* expression is linked to infants with high birth weight. Decreased *MEST* gene expression is observed in premature embryos compared to normal embryos, but does not affect DNA methylation (108).

MATERNAL STRESS AND MITOCHONDRIA

Moving from single genes to subcellular functional systems, converging lines of evidence have pointed to an important role of mitochondria, the traditional "powerhouses of the cell," as regulators of the stress response (109–111). Given the maternal origin and inheritance of mitochondria, it is plausible that maternal stress may exercise its effects on the offspring *via* alterations of mitochondrial pathways in both the *in utero* maternal microenvironment and offspring. Along these lines, it has been shown that maternal prenatal stress affects mitochondrial protein expression in pathways related to mitochondrial biogenesis and energy production in PFC and hippocampus of male rat offspring (112). Early-life maternal deprivation leads to a decrease in mitochondrial-related muscle gene expression in adult rats. Interestingly, adult-onset chronic stress had no effect on mitochondrial-related muscle gene expression function, indicating an early-life stress-specific effect (113). In humans, maternal psychosocial stress has been reported to alter the expression of mitochondrial proteins in the placenta (114). In this study, a link between mitochondrial changes and infant temperament has also been suggested. Maternal psychosocial stress and lifetime trauma have been associated with decreased mitochondrial DNA copy number in the placenta (115, 116).

INDIVIDUALITY

Chronic stress links changes in the epigenetic landscape with health conditions (117). Different cell types are characterized by distinct patterns of gene expression due to developmental, environmental, physiological, and pathological reasons (117). Epigenetic mechanisms affect gene function in a dynamic way as a result of different environmental exposures during fetal development. Early-life stressful experiences, such as nutritional deprivation, lack of maternal care, or chemical exposure during critical developmental periods, can lead to phenotypic differences later in life (118). In addition to genetic susceptibility (polymorphisms, genomic architecture) inter-individual phenotypic variations are also the result of epigenetic modifications. Once we realize how different environmental triggers affect the individual epigenetic processes, we may be able to develop new means to prevent or reverse environmentally driven epigenetic changes. A recent study supports this theory and suggests that adaptation to stress is a combination of three important factors: genetic predisposition, early-life environment, and late-life environment (119). In animal models, strain, age, sex, frequency, and duration of the stressor, time point within the light cycle and temperature, and even the housing conditions are some of the environmental factors that shape the stress response(120–122). In humans, genetic background, age, sex, type, frequency, and duration of the stressor and developmental stage have been suggested to be important factors that shape individual stress response (123).

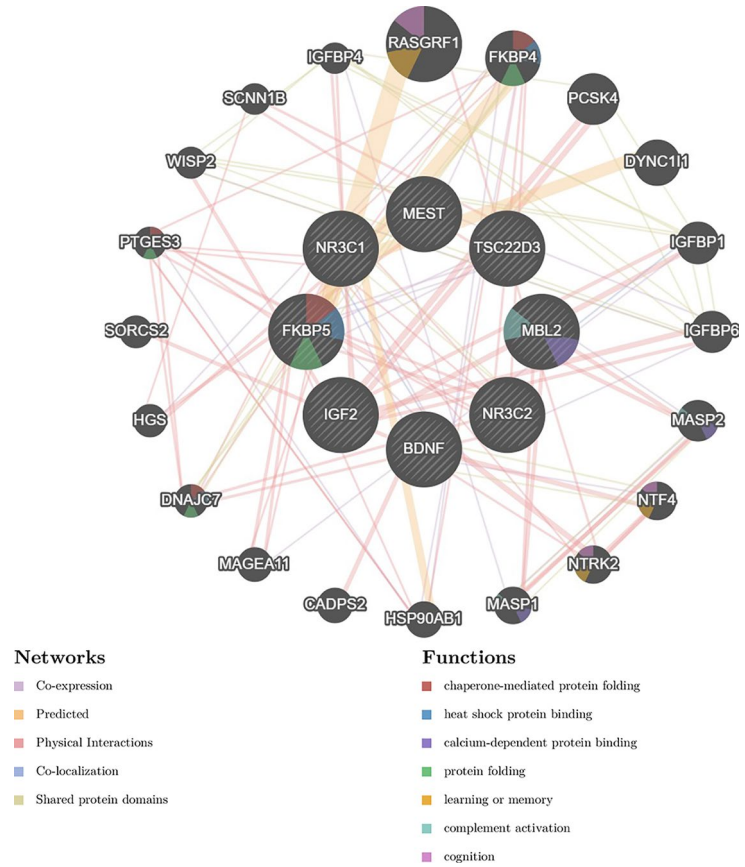

FIGURE 2 | Gene interaction network discussed in the current review. The network was generated by the GeneMANIA prediction server (124). The left panel presents the different types of interactions with respective color coding, depicted with lines connecting genes in the network: physical interactions (pink), predicted (orange), co-expression (purple), and shared protein domains. The right panel presents gene functions, depicted with colored slices inside the respective genes: chaperone-mediated protein folding (red), heat shock protein binding (blue), calcium dependent protein binding (dark blue), protein folding (green), learning of memory (orange), complement activation (light blue), cognition (purple).

DISCUSSIONS PERSPECTIVES

Early-life stress can influence brain plasticity with lasting effects. Epigenetic factors including type of exposure, timing, and diversity of experience in combination with genetic predisposition contribute to the individual resilience or vulnerability toward stress. Elucidating the interplay and downstream affected pathways (**Figure 2**) among i) housekeeping genes of the reproductive system, ii) regulators of the HPA axis, iii) components of mitochondrial heterogeneity, and iv) individual genomic architecture will facilitate our understanding of the impact of early-life stressful events for later life outcomes. Our analysis reveals the top 20 "satellite" genes (**Figure 2**) that form a functional network, affecting and being affected by the core genes controlling early-life stress. Potentially stressful or compensatory individual experiences during lifetime may have an impact on the epigenetic landscape, thus masking the effects of early-life experiences. An improved understanding will allow an integrated, systemic approach to address pathological stress responses and pinpoint novel molecular targets for pharmacological and therapeutic interventions.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of the manuscript.

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Adenosine-to-Inosine RNA Editing Within Corticolimbic Brain Regions Is Regulated in Response to Chronic Social Defeat Stress in Mice

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Adenosine-to-inosine (A-to-I) RNA editing is a co-/posttranscriptional modification of double-stranded RNA, catalyzed by the adenosine deaminase acting on RNA (ADAR) family of enzymes, which results in recognition of inosine as guanosine by translational and splicing machinery causing potential recoding events in amino acid sequences. A-to-I editing is prominent within brain-specific transcripts, and dysregulation of editing at several well-studied loci (e.g., *Gria2*, *Htr2c*) has been implicated in acute and chronic stress in rodents as well as neurological (e.g., Alzheimer's) and psychopathological disorders such as schizophrenia and major depressive disorder. However, only a small fraction of recoding sites has been investigated within the brain following stress, and our understanding of the role of RNA editing in transcriptome regulation following environmental stimuli remains poorly understood. Thus, we aimed to investigate A-to-I editing at hundreds of loci following chronic social defeat stress (CSDS) in mice within corticolimbic regions responsive to chronic stress regulation. Adult male mice were subjected to CSDS or control conditions for 21 days and dynamic regulation of A-to-I editing was investigated 2 and 8 days following the final defeat within both the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA). Employing a targeted resequencing approach, which utilizes microfluidics-based multiplex polymerase chain reaction (PCR) coupled with next-generation sequencing, we analyzed A-to-I editing at ~100 high-confidence editing sites within the mouse brain. CSDS resulted in acute regulation of transcripts encoding several ADAR enzymes, which normalized 8 days following the final defeat and was specific for susceptible mice. In contrast, sequencing analysis revealed modest and dynamic regulation of A-to-I editing within numerous transcripts in both the mPFC and BLA of resilient and susceptible mice at both 2 and 8 days following CSDS with minimal overlap between regions and time points. Editing within the *Htr2c* transcript and relative abundance of *Htr2c* messenger RNA (mRNA) variants were also observed within the BLA of susceptible mice 2 days following CSDS. These results indicate dynamic RNA editing within discrete brain regions following CSDS in mice, further implicating A-to-I editing as a stress-sensitive molecular mechanism within the brain of potential relevance to resiliency and susceptibility to CSDS.

Keywords: A-to-I RNA editing, chronic social defeat stress, microfluidics-based multiplex polymerase chain reaction, adenosine deaminases acting on RNA, ADAR, chronic stress

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INTRODUCTION

Adenosine-to-inosine (A-to-I) RNA editing is a co-/posttranscriptional modification of double-stranded RNA (dsRNA), which is catalyzed by the adenosine deaminases acting on RNA (ADAR) family of enzymes and is the most abundant form of RNA editing in higher eukaryotes (1). ADAR enzymes deaminate adenosine bases to inosine, which is recognized as guanosine by ribosomes and splicing machinery. As such, RNA editing can induce nonsynonymous amino acid changes resulting in differential protein isoform expression and thus is considered a key mechanism of transcriptome and proteome diversification in metazoans (2).

Three members of the ADAR family are encoded in the mammalian genome including the catalytically active ADAR (ADAR1) and ADARB1 (ADAR2), as well as ADARB2 (ADAR3), which lacks a catalytic domain and is primarily restricted to low-level expression within the brain (3). Furthermore, ADAR has two distinct splicing isoforms including the constitutive 110-kDa isoform ADAR1 (p110) and the interferon-inducible isoform ADAR (p150) (4). ADARs mediate RNA editing at millions of sites in the mammalian transcriptome in both coding and noncoding RNA. Recent evidence suggests that ADAR primarily mediates A-to-I editing at repetitive elements, such as *Alu* repeats in primates, where ADARB1 is primarily responsible for editing at coding sites, although a degree of overlap exists between targeted sites of the two enzymes within mammalian tissues (5). Although several functions are known for editing events in noncoding sites [e.g., alteration of microRNA (miRNA) binding to 3′untranslated regions (UTRs) and alternative splicing regulation] (6–8), much interest has been focused on editing sites within coding regions capable of inducing nonsynonymous recoding events. These events are appreciated as a common form of proteome diversification in both basal and pathological states (9). Interestingly, such recoding events are enriched within the brain and also reside more commonly in transcripts associated with brain function, such as those encoding ion channels and neuromodulator receptors (10). For example, well-established ADARB1-dependent editing of the *Gria2* transcript encoding the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit Glutamate Ionotropic Receptor AMPA Type Subunit 2 (GRIA2) is essential for normal development as *Adarb1* knockout mice die within 3 weeks of birth. This can be rescued upon transgenic coexpression of the fully edited *Gria2* isoform in these mice (11). Another well-established role of RNA editing within the mammalian brain is regulation of the 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor as multiple recoding sites in the *Htr2c* transcript generates multiple 5-HT$_{2C}$ receptor isoforms with varying G protein affinities and thus receptor function (12).

RNA editing within the brain is also sensitive to environmental and pharmacological stimuli as acute and chronic stress as well as antidepressant treatment in rodents dynamically regulates *Htr2c* editing and thus serotonergic signaling within discrete brain regions (13, 14). Moreover, editing of the *Htr2c* transcript is observed within both the dorsolateral prefrontal cortex (PFC) (BA9) and anterior cingulate cortex (BA24) of patients with major depressive disorder (MDD), indicative of the translational relevance of investigating stress-induced regulation of well-conserved editing sites in rodent models of acute and chronic stress (15, 16). Despite this, our understanding of the stress-induced changes in the RNA editome remain restricted to well-studied candidate loci. Broader high-throughput approaches are necessary to identify novel stress-sensitive editing sites within the brain of potential relevance to stress-related psychiatric disorders.

Recent advancements in next-generation sequencing (NGS) technologies have significantly enhanced our ability to accurately quantify A-to-I editing throughout the transcriptome and broadened our understanding of aberrant A-to-I editing in several neurological diseases (e.g., Alzheimer's disease, epilepsy, and amyotrophic lateral sclerosis) (17–19) and psychiatric disorders (schizophrenia, bipolar disorder, and autism spectrum disorder) (20). However, application of NGS-based techniques in rodent models of acute and chronic stress is lacking such that our understanding of the role of RNA editing in acute and long-term adaptations to stress remains poorly understood.

Thus, this study aimed to investigate aberrant RNA editing within corticolimbic brain regions following chronic social defeat stress (CSDS) in adult mice. CSDS is a well-characterized model of depression-like behavior with significant etiological, predictive, discriminative, and face validity. CSDS induces diverse transcriptome changes within corticolimbic circuits such as the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA) thought to subserve stable behavioral deficits in this model (21, 22). We therefore hypothesized that CSDS would induce dynamic changes within the RNA editome within the mPFC and BLA. To identify novel stress-sensitive editing sites within these brain regions, we employed an established microfluidics-based multiplex polymerase chain reaction and deep sequencing (mmPCR-seq) approach (19, 23) to accurately quantify RNA editing at hundreds of loci within the brain following CSDS.

METHODS

Animals

Male C57BL/6 mice at 10 to 12 weeks old were employed for all experiments (Charles River, Sulzfeld, Germany). Mice were single housed in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany, for 1 week prior to experimentation and were maintained under standard conditions (12L:12D light cycle, lights on at 07:00 AM, temperature 23 ± 2°C) with food and water available *ad libitum*. All experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee (Government of Upper Bavaria, Munich, Germany).

Chronic Social Defeat Stress

Mice were randomly assigned to stress (n = 25) or control conditions (n = 16) and were subjected to 21 days of social defeat or daily handling, respectively. CSDS was conducted essentially as previously described (24). Briefly, male CD1 mice (16 to 18 weeks of age) were employed as resident mice and were habituated to the

social defeat cage for 1 week prior to experimentation. On each defeat day, experimental mice were introduced into the home cage (45 cm × 25 cm) of a dominant resident mouse, and mice were left until a social defeat was achieved with minimal injury to experimental mice (generally >2 min). Once mice were defeated, the animals were separated by a wire mesh to prevent physical contact while enabling sensory contact for 24 h. Experimental mice were defeated daily by an unfamiliar, resident mouse for 21 days between 9:00 and 16:00 h to minimize habituation to the CSDS procedure. Control mice (Con) were singly housed in their home cage and handled daily throughout the CSDS procedure. Immediately following the final defeat, experimental mice were single housed. One day following the final defeat, all mice were subjected to the social interaction (SI) test to enable classification of susceptible (SUS) [social interaction (SI) ratio <1] and resilient mice (RES) (SI ratio >1) as previously described (22, 24). Following the SI test, all mice remained in their home cage undisturbed until tissue collection either 2 days (control, $n = 8$; susceptible, $n = 10$; resilient, $n = 4$) or 8 days (control, $n = 8$; susceptible, $n = 8$; resilient, $n = 3$) following the final defeat.

Tissue Collection and Corticosterone Analysis

Either 2 or 8 days following the final defeat, mice were sacrificed (9:00–10:00) and brains were rapidly dissected, snap frozen, and stored at −80°C. Bilateral adrenal glands were dissected, cleaned of excess fat, and weighed. Whole blood was collected in Ethylenediaminetetraacetic acid tubes and centrifuged at 8,000 × g for 10 min at 4°C. Plasma was then aliquoted and stored at −20°C until corticosterone analysis, employing a commercially available radioimmunoassay kit (MP Biomedicals Inc).

Brain Region Microdissection, RNA Extraction, and Reverse Transcription

Frozen brains were serially sectioned at 250 µm on a cryostat, and the mPFC (including prelimbic, infralimbic, and cingulate cortex) and BLA were microdissected and stored at −80°C. Total RNA was extracted from mPFC and BLA tissue from each animal utilizing the miRNeasy mini kit (QIAGEN). Briefly, tissue punches were lysed in 700 µl of Qiazol, and total RNA was extracted as per manufacturer's instructions (QIAGEN). RNA integrity was assessed on an Agilent Tapestation 2200 with the RNA screentape kit (Thermo Fisher), and all RNA samples were confirmed to have RNA integrity number (RIN) values > 8. Total RNA was DNase treated with the TURBO DNase free kit as per manufacturer's instructions (Ambion). DNase-treated RNA was quantified with the Qubit 3.0 (Thermo Fisher), and 200 ng of RNA was reverse transcribed to cDNA in 20-µL reactions employing the iScript™ cDNA Synthesis Kit as per manufacturer's instructions (Bio-Rad). Complementary DNA (cDNA) was stored at −20°C prior to further analysis. Due to failure of cDNA synthesis for multiple samples, final group sizes analyzed for RNA editing and quantitative PCR (qPCR) analysis were as follows: control 2 days, $n = 6–8$; susceptible 2 days, $n = 10$; resilient 2 days, $n = 4$; control 8 days, $n = 5–8$; susceptible 8 days, $n = 7–8$; resilient 8 days, $n = 3$.

Quantitative Real-Time Polymerase Chain Reaction

Determination of relative transcript expression was conducted using the $2^{-\Delta\Delta CT}$ method (25, 26). Exon spanning primers for candidate transcripts *Adar*, *Adar variant 2*, *Adar variant 3*, *Adarb1*, *Adarb2*, *Nova1*, *Commd2*, *Gria4*, and *Htr2c* and endogenous controls *Gapdh*, *Rpl13a*, and *Sdha* were designed using Primer 3 (http://frodo.wi.mit.edu/). Primer efficiencies were confirmed to be 90–110%. qPCR was conducted on a Quantistudio Flex7 PCR system (Applied Biosystems, USA) using Quantifast SYBR® Green (QIAGEN) as per manufacturer's instructions. All data are expressed as fold change relative to control mice at each time point.

Targeted Resequencing of RNA Editing Sites in RNA Samples Using the Fluidigm Access Array Coupled With Illumina HiSeq 2500 Sequencing

To precisely detect and measure the levels of A-to-I RNA editing at candidate editing sites in mouse brain tissue, we employed a targeted resequencing approach utilizing mmPCR-seq essentially as previously described (19). Candidate editing sites were selected from the mouse RADAR database (v.2; http://rnaedit.com/) based on the following criteria: i) location within protein coding genes, ii) induction of nonsynonymous amino acid changes, iii) species conservation, and iv) location within genes associated with neuronal function. Applying these criteria, we generated a candidate list of 551 editing sites for which targeted gene and editing-site-specific exon spanning primers were designed using Primer 3.0 (http://frodo.wi.mit.edu/). Selected primers were tested for specificity and sensitivity by PCR prior to their inclusion to the finalized primer set (**Supplemental Table 1**). Amplification of target regions containing targeting editing loci was conducted with the Access Array™ System for Illumina Sequencing Systems as per manufacturer's instructions. Briefly, 4 µL of single primer pair (4 µM per primer in 1× AA loading buffer) was loaded into the primer inlets of the 48.48 Access Array integrated fluidic circuits (IFC) (Fluidigm). To prepare the cDNA templates, 2.25 µL of each cDNA sample was added to 2.75 µL of presample mix containing the following enzyme and reagents from the Roche FastStart High Fidelity PCR System: 0.5 µL of 10× FastStart High Fidelity Reaction Buffer wo/Mg, 0.5 µL of dimethyl sulfoxide (DMSO) (5%), 0.1 µL of 10 mM PCR Grade Nucleotide Mix (200 µM), 0.9 µL of 25 mM $MgCl_2$ (4.5 mM), 0.25 µL of 20× Access Array Loading Reagent (Fluidigm), 0.05 µL of FastStart High Fidelity Enzyme Blend, and 0.7 µL of PCR grade water. Four microliters of this mix was loaded into the sample inlets of the 48.48 Access Array IFC (Fluidigm). After the loading of both samples and primers *via* IFC Controller AX (Fluidigm) loading script, the IFC was subject to thermal cycling using the FC1 Cycler (Fluidigm) with the following program for 40 cycles: 50°C for 2 min, 70°C for 20 min, 95°C 10 min; 10 cycles of 95°C for 15 s, 59.5°C for 30 s, 72°C for 1 min; 4 cycles of 95°C for 15 s, 80°C for 30 s, 59.5°C for 30 s, 72°C for 1 min; 10 cycles of 95°C for 15 s, 59.5°C for 30 s, 72°C for 1 min; 4 cycles of 95°C for 15 s, 80°C for 30 s, 60°C for 30 s, 72°C for 1 min; 8 cycles of 95°C for 15 s, 59.5°C for

30 s, 72°C for 1 min; 4 cycles of 95°C for 15 s, 80°C for 30 s, 60°C for 30 s; 72°C for 1 min; finalizing with 72°C for 3 min. Once PCR has terminated, the IFC was transferred to another IFC Controller AX (Fluidigm) and mini-libraries were harvested by the controller harvest script. Thus, mini-libraries from each sample were obtained for further barcoding and sequencing.

Sequencing Adaptor and Barcode Addition

For each sample, 1.0 μL of the PCR products harvested from the IFC was 1:110 diluted and added to 15 μL of presample mix containing the following enzyme and reagents from the Roche FastStart High Fidelity PCR System: 2 μL of 10× FastStart High Fidelity Reaction Buffer wo/Mg, 1 μL of DMSO (5%), 0.4 μL of 10 mM PCR Grade Nucleotide Mix (200 μM), 3.6 μL of 25 mM $MgCl_2$ (4.5 mM), 0.2 μL of FastStart High Fidelity Enzyme Blend, and 7.8 μL of PCR-grade water. To that samples mix, 4 μL of primer mix from the 2 μM Access Array Barcode Library for Illumina Sequencer—384 (Fluidigm, PN 100-3771), utilizing the B-set; PE2_BC_CS2 and PE1–CS1 barcode primer combination. We used the following PCR program: 95°C for 10 min; 10 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. Amplified libraries were purified with AMPure XP beads (Beckman Coulter) and subjected to paired-end 100-bp sequencing on an Illumina Hiseq2500.

Bioinformatics Sequence Analysis

Bioinformatics analysis was conducted essentially as previously described (19). Briefly, the University of California, Santa Cruz (UCSC) mouse genome browser (NCBI37/mm9) assembly is used to identify any A/G mismatches within the target cDNA sequences. Such mismatches were summed and calculated for their signal strength according to the overall number of reads coverage and the percentage of A-to-G levels.

Prealignment Processing

Fastq files were deindexed into 48 samples according to the individual barcodes used by an in-house script. All reads were trimmed of the universal CS1 and CS2 sequences, and short reads (<20 nt) were removed. Alignment of the processed reads was made using bwa version 0.7.4-r385, using the mem option and the parameters -k 20 -B 3 -O 3 -T 20 for seed in the length of the average primer and for considering the Ion typical error of small indel.

Alignment Process

Sequences were aligned to the mouse refseq database, and reads aligning to multiple loci were excluded from further analysis. Samtools mpileup was employed for aligned sequencing reads, and in-house scripts were employed to transfer the results to the genomic locations from the refseq loci followed by counting the number of different nucleotides in each genomic location that had a *q* score ≥20. To obtain high-confidence editing sites, only loci with >2,000 reads were included for further analysis, resulting in a total of 100 sites within the BLA and 105 sites within the mPFC (**Supplemental Table 2**). Data presented for each editing loci represent the total number of reads, and the calculated percentage of reads that have a "G" at the specified genomic location was conducted accordingly with the formula (# of "G" reads/[# of "G" reads + # of "A" reads]).

Statistical Analysis

Two-way repeated-measures analysis of variance (ANOVA) with Sidak *post hoc* comparison was used to compare body weights throughout the CSDS procedure and SI time in the SI test between control and stressed mice. One-way ANOVAs with Sidak *post hoc* comparisons were used to compare SI ratios between control, susceptible, and resilient mice as well as between groups at each time point for body weights at sacrifice, adrenal weights, corticosterone levels, qPCR expression levels, *Htr2c* editing, and isoform abundance. Editing levels were roughly normally distributed, and no normalization was applied such that paired *t* tests were employed for each RNA editing site. Significance was accepted as $p < 0.05$. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

RESULTS

Adult male mice were subjected to 21 days of CSDS with no significant differences in body weight observed throughout the stress procedure as revealed by a main effect of time [$F_{(3,117)} = 45.83$, $p < 0.001$] but not of treatment or an interaction between factors (**Figure 1A**). CSDS significantly decreased SI with a novel CD1 mouse as evidenced by a main effect of trial [$F_{(1,37)} = 5.252$] and a treatment by trial interaction [$F_{(2,37)} = 14.47$] whereby susceptible mice spent significantly decreased time in the interaction zone in the CD1 compared to the habituation trial ($p < 0.001$, **Figure 1B**). For the SI ratio, there was a main effect of treatment [$F_{(2,37)} = 13.72$, $p < 0.001$] and significantly decreased SI ratio in susceptible mice and an increased SI ratio in resilient mice compared to controls ($p < 0.05$, **Figure 1C**). There were no differences in body weight between groups 2 or 8 days following the final defeat (**Figure 1D**). However, CSDS induced adrenal hypertrophy as evidenced by a main effect of treatment at 2 days [$F_{(2,19)} = 5.42$, $p = 0.014$] with significantly increased adrenal weights in both susceptible and resilient mice compared to controls at 2 days ($p < 0.01$) but not 8 days [$F_{(2,16)} = 2.524$, $p = 0.111$] following the final defeat (**Figure 1E**). No differences in basal corticosterone were observed at either time point following CSDS (**Figure 1F**).

As stress is known to alter A-to-I editing and ADAR levels within the rodent brain, we initially analyzed mRNA expression of transcripts encoding ADAR enzymes within the BLA and mPFC following CSDS. No significant difference in Adar mRNA expression levels was observed in the BLA 2 days following the final defeat, although a trend toward increased Adar transcript variant 3 mRNA encoding the shorter ADAR1 p110 protein and Adarb1 mRNA expression was evident in both susceptible and resilient mice, yet this did not reach statistical significance (**Figure 2A**). In contrast, CSDS induced a significant decrease in Adar transcript variant 2 mRNA ($p = 0.018$) encoding the longer ADAR1 p150 protein and Adarb1 mRNA expression ($p = 0.002$) specifically in the mPFC of susceptible mice 2 days following the

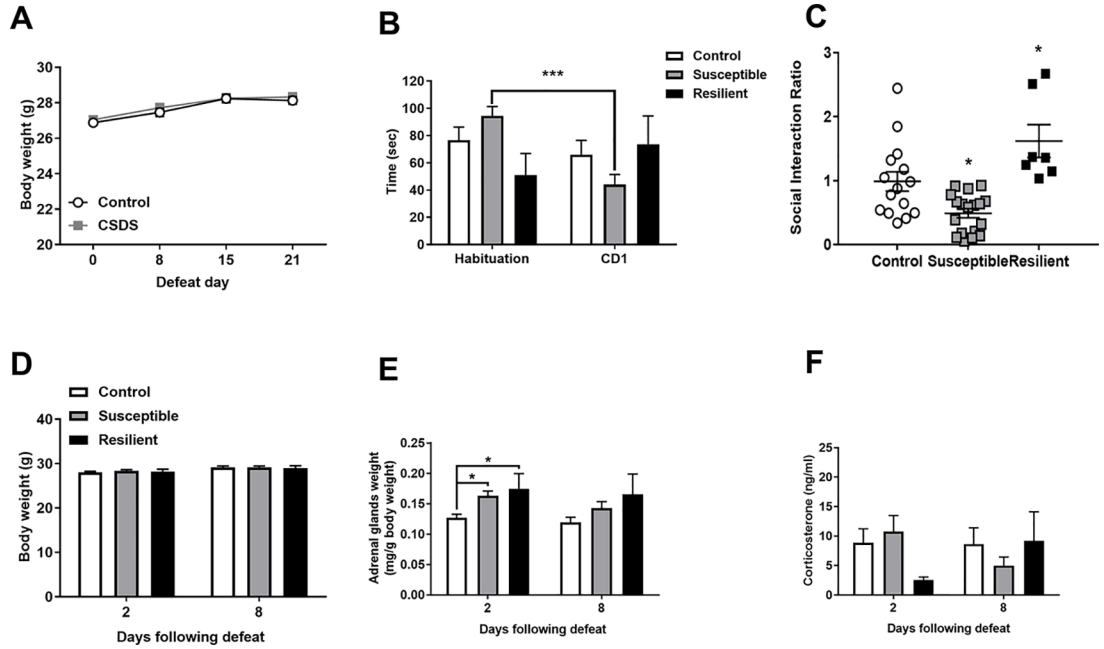

FIGURE 1 | Chronic social defeat stress (CSDS) induces social avoidance and adrenal hypertrophy without changes in basal corticosterone levels. Body weight throughout the stress procedure did not differ between groups **(A)**. Following CSDS, susceptible mice spent significantly less time investigating a novel CD1 mouse than an empty cage in the social interaction (SI) test **(B)** and significantly decreased the SI ratio in susceptible and increased SI ratio in resilient mice compared to controls **(C)**. CSDS did not alter body weight at 2 or 8 days following the final defeat **(D)** but induced an increase in adrenal weight in both susceptible and resilient mice at 2 days but not 8 days **(E)** with no changes in basal corticosterone **(F)**. ***$p < 0.001$, two-way repeated measures (RM) ANOVA with Sidak *post hoc* comparisons; *$p < 0.05$, susceptible and resilient groups compared to control, one-way ANOVA with Sidak *post hoc* comparisons.

final defeat (**Figure 2C**). No differences in expression levels of Adar mRNAs were observed 8 days following CSDS (**Figure 2**).

To assess CSDS-induced A-to-I RNA editing within the mPFC and BLA, we employed an established mmPCR-seq approach for sensitive and accurate quantification of RNA editing (19). Employing high-confidence editing sites (see Methods), we quantified editing at 100 sites within the mouse BLA and 105 sites within the mouse mPFC. Sequencing analysis revealed that global levels of A-to-I editing within the BLA did not differ at either 2 days [Control mice (Con), 22.96% ± 0.23%; susceptible mice (SUS), 23.10% ± 0.46%; resilient mice (RES), 23.06% ± 0.33%] or 8 days (Con, 23.42% ± 0.29%; SUS, 23.09% ± 0.08%; RES, 23.39% ± 0.19%) following CSDS. Similarly, no differences were observed in the mPFC at 2 days (Con, 24.65% ± 0.24%; SUS, 24.66% ± 0.34%, RES, 25.03% ± 0.40%) or 8 days following CSDS (Con, 24.93% ± 0.25%; SUS, 24.65% ± 0.20%; RES, 24.62% ± 0.34%). Considering the lack of differences in global editing levels between groups, we pooled all samples in each region and found a significantly increased global editing level within the mPFC (24.74% ± 0.12%) compared to BLA (23.20% ± 0.14%, $p < 0.001$). This finding and global editing levels are in line with those previously reported for the rodent brain with mmPCR-seq (5, 27).

In contrast, differential A-to-I editing was observed at four editing sites in susceptible mice and one site in resilient mice within the BLA 2 days following CSDS with another seven differentially edited sites identified in susceptible mice and six sites in resilient mice 8 days following CSDS within this region (**Table 1**). Dynamic regulation of editing within the *Zfp324* transcript was observed within the BLA of susceptible mice with decreased and increased editing observed at 2 and 8 days, respectively. No other persistent changes were observed in this region. Within the mPFC, four sites were differentially edited in susceptible mice and another four sites were differentially edited in resilient mice 2 days following CSDS with a further seven sites in susceptible mice and four sites in resilient mice differentially edited 8 days following CSDS (**Table 2**). Increased editing at the *Commd2* locus encoding the Copper metabolism Murr1 domain-containing protein 2 (COMMD2) was observed at both 2 and 8 days following CSDS with no other persistent changes observed. Moreover, RNA editing of a nonsynonymous recoding site within the *Nova1* transcript encoding the RNA binding protein (RBP) NOVA Alternative Splicing Regulator 1 (NOVA1) was also observed in both the BLA and mPFC 8 days following CSDS, although in opposite directions. Differential editing of several sites following CSDS did not alter mRNA expression levels of candidate transcripts in either brain region (**Figure 3**).

Well-established A-to-I editing of five sites within the *Htr2c* transcript, known as A, B, C, D, and E, results in the generation of 32 mRNA variants generating up to 24 different protein isoforms of the HTR2C receptor with varying biochemical properties (28). As expected, we detected high-confidence editing at sites A, B, C, and D but only minimal editing at site E within the mouse BLA and mPFC (**Figure 4**). CSDS induced a modest increase in editing at both the C and D sites within the BLA of susceptible

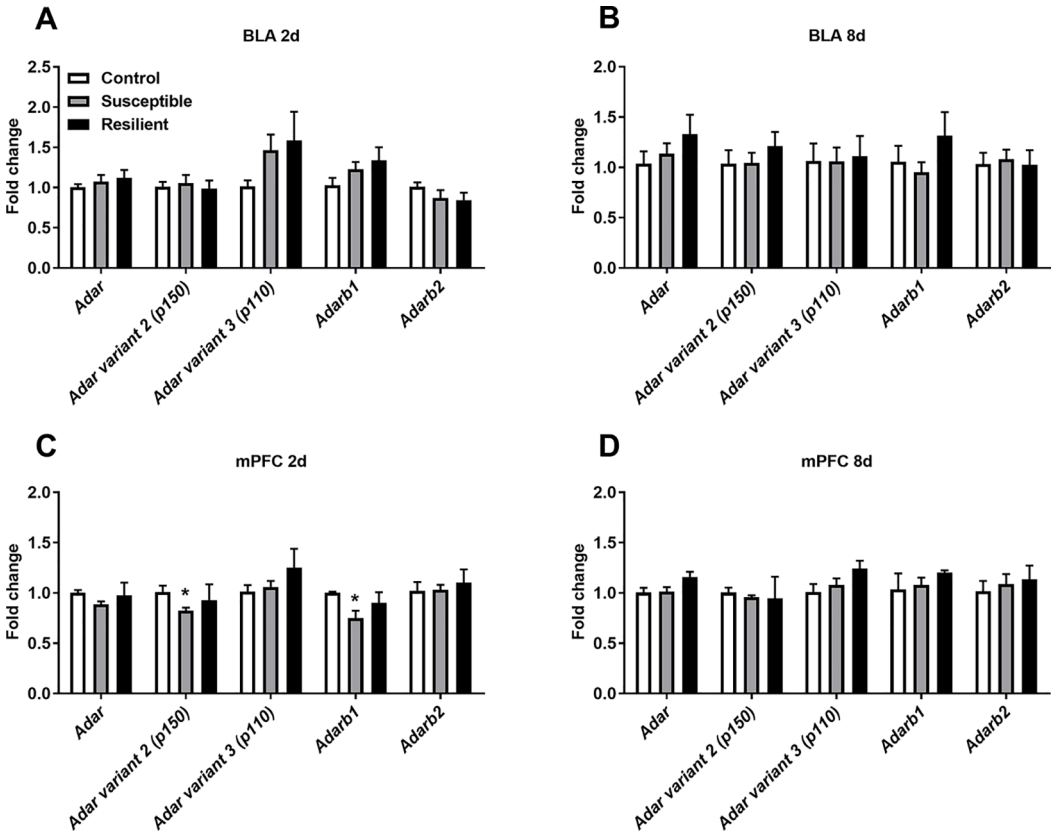

FIGURE 2 | CSDS acutely alters transcripts encoding adenosine deaminases acting on RNA (ADARs) within the medial prefrontal cortex (mPFC) of susceptible mice. Following CSDS, no changes in Adar, Adarb1, or Adarb2 mRNA levels were observed within the basolateral amygdala (BLA) at 2 days **(A)** or 8 days **(B)**. CSDS significantly decreased the expression levels of Adar variant 2 (p150) and Adarb1 mRNA in the mPFC only in mice susceptible to the CSDS procedure **(C)**. No changes were observed within the mPFC 8 days following CSDS **(D)**. Control 2 days, $n = 7$; susceptible 2 days, $n = 10$; resilient 2 days, $n = 4$; control 8 days, $n = 5$–7; susceptible 8 days, $n = 7$; resilient 8 days, $n = 3$. $^*p < 0.05$, one-way ANOVA with Sidak *post hoc* comparisons.

mice but not resilient mice 2 days following the final defeat (**Figure 4A**) with no other changes observed at 8 days (**Figure 4B**) or within the mPFC at either time point (**Figure 4C** and **D**).

Editing at these loci induces recoding events, ultimately generating different HTR2C protein isoforms. Thus, we next quantified the relative abundance of *Htr2c* mRNA variants within the BLA and mPFC following CSDS. As expected, there was a similar distribution of *Htr2c* mRNA variants within the BLA and mPFC, with the edited VNV isoform being most abundant in both regions (**Figure 5**), as previously reported in the rodent brain (27). Following CSDS, we observed a trend toward an effect of treatment for the VNI variant [$F_{(2,18)} = 3.068$, $p = 0.071$] mainly due to a trend toward decreased VNI abundance in susceptible mice at this time point ($p = 0.058$, **Figure 5A**). No other changes were observed within the BLA at 8 days (**Figure 5B**) or within the mPFC (**Figure 5C** and **D**).

DISCUSSION

In this study, we demonstrated that CSDS in adult mice induces a moderate degree of differential editing in a subset of novel transcripts within the BLA and mPFC, including modest regulation of editing within the *Htr2c* transcript and thus isoform abundance previously demonstrated to be sensitive to stress-induced regulation. Our results further emphasize the sensitivity of RNA editing to stress and suggest that both acute and chronic changes in editing, although moderate, may contribute to behavioral deficits observed following CSDS in adult mice. Moreover, differential regulation in susceptible and resilient mice suggests that RNA editing may be a novel molecular mechanism involved in resiliency and susceptibility in this model requiring further investigation.

To our knowledge, this is the first study to investigate a large subset of RNA editing sites within the mouse brain in response to chronic stress and the first report of A-to-I editing following CSDS. Interestingly, minimal changes in transcripts encoding ADAR enzymes were identified following CSDS. Specific reduction of the interferon-inducible Adar variant 2 (p150) and Adarb1 mRNA levels was observed within the mPFC of susceptible mice only without changes in global RNA editing in these mice. RNA editing within the rodent brain is relatively stable upon induction of ADAR p150, such that decreased levels within the mPFC are unlikely to explain observed effects in this study (29).

TABLE 1 | Differentially editied loci within the basolateral amygdala (BLA) following chronic social defeat stress (CSDS).

Gene name	Edit site location	Edit % CON	SEM CON	Edit % SUS	SEM SUS	Edit % difference SUS	*p* value SUS	Edit % RES	SEM RES	Edit % difference RES	*p* value RES
BLA 2 d CON vs. SUS											
Htr2c (site C)	chrX:143604236	22.902	0.958	25.718	0.317	2.816	0.005	24.957	0.234	2.055	0.128
Htr2c (site D)	chrX:143604241	58.005	0.627	60.312	0.572	2.306	0.021	59.048	0.653	1.043	0.299
Zfp324	*chr7:13557536*	16.870	1.008	14.172	0.635	–2.698	0.031	13.263	1.282	–3.607	0.056
Gabra3	chrX:69690631	89.342	0.362	90.279	0.246	0.937	0.044	89.567	0.634	0.226	0.747
BLA 2 d CON vs. RES											
Gla	*chrX:131123629*	3.590	0.736	5.317	0.909	1.727	0.210	6.121	0.689	2.530	0.046
BLA 8 d CON vs. SUS											
Zfp324	*chr7:13557536*	13.305	0.557	15.761	0.582	2.456	0.009	13.596	2.069	0.291	0.849
Copa	chr1:174022479	4.663	0.100	4.299	0.081	–0.364	0.016	4.556	0.074	–0.107	0.552
Tcp11l1	*chr2:104521242*	16.440	0.616	18.758	0.567	2.318	0.017	17.583	0.451	1.143	0.313
Gria4	chr9:4456006	91.448	1.531	86.767	1.210	–4.680	0.035	87.457	0.697	–3.991	0.161
Fubp3	*chr2:31471414*	6.945	0.257	6.087	0.273	–0.858	0.040	7.249	0.289	0.303	0.528
Qpctl	*chr7:19725738*	9.246	0.284	10.356	0.406	1.110	0.040	9.674	0.366	0.429	0.432
Nova1	chr12:47801321	12.654	0.441	11.435	0.321	–1.218	0.049	10.818	0.201	–1.835	0.038
BLA 8 d CON vs. RES											
Bri3bp	*chr5:125936975*	74.982	0.324	73.838	0.957	–1.144	0.253	77.648	0.162	2.665	0.001
Slc35e1	*chr8:75004254*	23.534	0.452	24.349	1.537	0.815	0.598	27.133	0.748	3.599	0.003
Samd8	*chr14:22616933*	25.989	0.501	27.677	1.143	1.688	0.180	29.333	1.179	3.344	0.012
Nt5dc3	*chr10:86299972*	2.464	0.085	2.340	0.089	–0.124	0.333	2.103	0.063	–0.361	0.037
Nup155	*chr15:8109489*	52.694	0.924	54.684	1.203	1.990	0.206	56.497	0.412	3.803	0.039

Microfluidics-based multiplex polymerase chain reaction and deep sequencing (mmPCR-seq) identified differential editing at numerous editing sites within the BLA at both 2 and 8 days following CSDS. No persistent changes in editing levels were observed at both 2 and 8 days following CSDS. Underlined edit site locations indicate RNA editing sites within exons. Italicized edit site locations indicate RNA editing sites within 3′UTRs. $p < 0.05$, Student's t test.
CON, control; SUS, susceptible; RES, resilient.

TABLE 2 | Differentially editied loci within the medial prefrontal cortex (mPFC) following CSDS.

Gene name	Edit site location	Edit % CON	SEM CON	Edit % SUS	SEM SUS	Edit % difference SUS	*p* value SUS	Edit % RES	SEM RES	Edit % difference RES	*p* value RES
mPFC 2 d CON vs. SUS											
Commd2	*chr3:57448409*	58.522	2.379	67.992	2.120	9.470	0.009	63.454	0.666	4.932	0.186
Rsad1	*chr11:94401990*	5.970	2.226	13.520	1.520	7.551	0.011	9.871	2.994	3.901	0.329
Wipi2	*chr5:143145189*	58.787	2.598	50.223	2.574	–8.564	0.034	53.321	1.579	–5.465	0.193
Zfp81	chr17:33472367	2.595	0.755	0.806	0.356	–1.789	0.036	1.347	1.079	–1.248	0.364
mPFC 2 d CON vs. RES											
Ncl	*chr1:88244312*	27.480	0.792	30.468	1.239	2.988	0.074	31.555	1.045	4.076	0.013
Snhg11	chr2:158209361	21.319	0.392	23.068	1.234	1.749	0.239	24.089	0.775	2.770	0.005
Acan	chr7:86242858	3.867	0.336	4.053	0.840	0.187	0.853	2.069	0.306	–1.798	0.007
Nt5dc3	*chr10:86299972*	2.380	0.070	2.367	0.230	–0.013	0.962	2.628	0.065	0.248	0.048
mPFC 8 d CON vs. SUS											
Rn45s	*chr17:39980697*	22.675	1.391	16.562	1.606	–6.113	0.017	20.696	0.906	–1.979	0.382
Iqgap1	*chr7:87856938*	4.055	0.938	8.971	1.192	4.916	0.009	4.685	2.215	0.630	0.762
Commd2	*chr3:57448409*	59.534	1.400	64.514	1.180	4.980	0.019	62.746	2.209	3.212	0.241
Klf16	*chr10:80030104*	9.441	0.346	10.741	0.329	1.300	0.020	10.037	0.560	0.595	0.372
Rwdd2b	*chr16:87434377*	17.327	1.261	14.255	0.554	–3.072	0.038	18.937	4.031	1.610	0.632
Nova1	chr12:47801321	10.549	1.160	13.241	0.374	2.691	0.038	12.090	0.446	1.541	0.401
Dagla	*chr19:10320223*	3.144	0.253	2.450	0.151	–0.695	0.033	2.536	0.153	–0.609	0.157
mPFC 8 d CON vs. RES											
Rabl5	*chr5:137388969*	16.523	0.454	15.679	0.455	–0.844	0.184	14.702	0.318	–1.821	0.045
Copa	chr1:174022479	4.310	0.140	4.301	0.096	–0.009	0.955	4.944	0.154	0.634	0.036
Dnajc18	*chr18:35834187*	12.818	0.434	13.277	0.512	0.459	0.528	14.915	0.687	2.098	0.025

Microfluidics-based multiplex PCR and deep sequencing (mmPCR-seq) identified differential editing at numerous editing sites within the PFC at both 2 and 8 days following CSDS. Increased editing at a site within the Commd2 transcript was observed at both 2 and 8 days following CSDS with no other persistent changes observed. Underlined edit site locations indicate RNA editing sites within exons. Italicized edit site locations indicate RNA editing sites within 3′UTRs. $p < 0.05$, Student's t test.
CON, control; SUS, susceptible; RES, resilient.

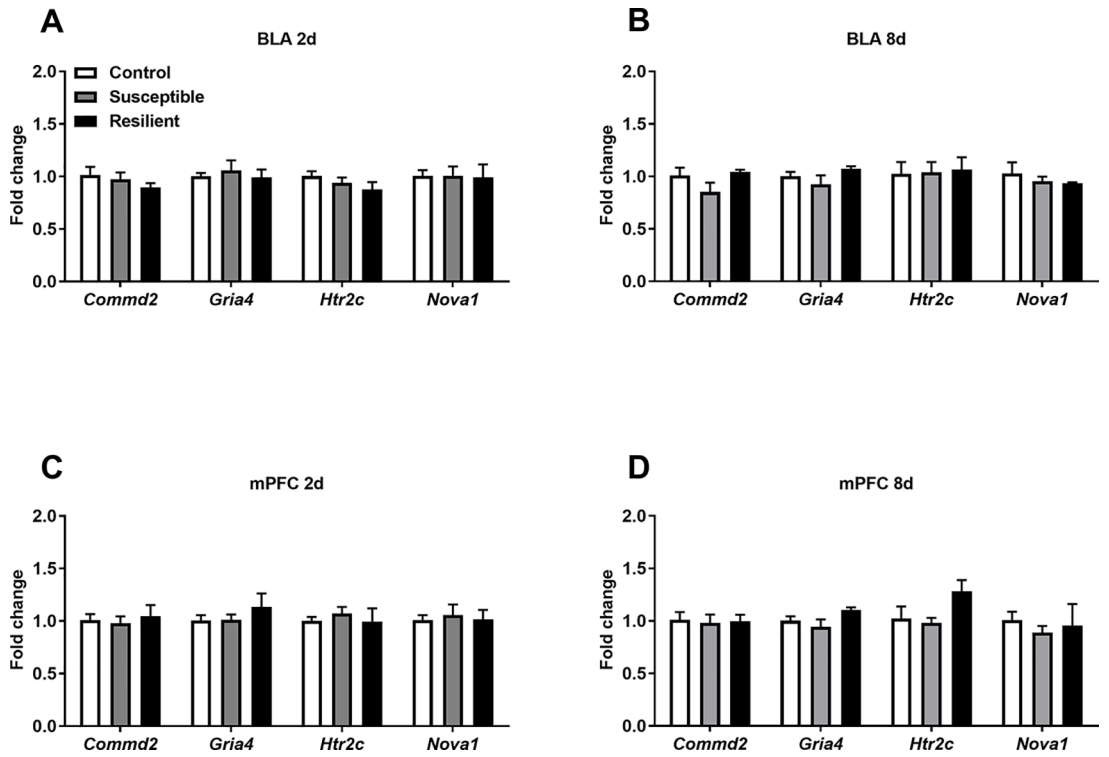

FIGURE 3 | Expression levels of differentially edited transcripts are unaltered following CSDS. No significant changes in the expression levels of several differentially edited transcripts were observed with the BLA **(A)** or mPFC **(B)** at 2 days or within the BLA **(C)** or mPFC **(D)** 8 days following the final defeat. Control 2 days, *n* = 7; susceptible 2 days, *n* = 10; resilient 2 days, *n* = 4; control 8 days, *n* = 5–7; susceptible 8 days, *n* = 7; resilient 8 days, *n* = 3.

As ADARB1 is primarily responsible for editing at recoding sites (5), decreased expression in susceptible mice may mediate decreased editing at specific ADARB1 target sites. However, we observed both increased editing within *Commd2* and *Rsad1* and decreased editing in *Wipi2* and *Zfp81* within the mPFC of susceptible mice. Moreover, editing differences were also observed following CSDS in the absence of Adar mRNA expression changes, suggesting that differential RNA editing is unlikely to be mediated by the Adarb1 mRNA expression changes observed. This is in line with evidence suggesting complex regulation of RNA editing activity in a tissue-specific and cell-type-specific manner independent of ADAR family expression levels including interaction with RBPs, such as fragile X mental retardation protein (2, 5, 30, 31). Thus, our results suggest that differential RNA editing at the sites identified in this study is more likely mediated by site-specific regulation of editing activity opposed to CSDS-induced changes in the levels of ADAR family enzymes.

Although we identified differential editing at novel editing sites within the mouse brain, notable differential editing was observed within the *Htr2c* transcript specifically within the BLA of susceptible mice. Considering the well-established regulation of editing within this transcript in rodent models of acute and chronic stress as well as within the brain of MDD patients (13–16, 32), these results further implicate editing at this transcript in susceptibility to CSDS and support the model's relevance to stress-related psychiatric disorders. However, it must be noted that stress-induced editing of the *Htr2c* transcript and variant abundance is context-dependent based on species, strain, stress modality, brain region, and developmental age (27, 28). RNA editing of the *Htr2c* transcript reduces both receptor/Gαq-protein coupling and constitutive activity of the $5HT_{2C}$ receptor (12, 33, 34). Transgenic mice exclusively expressing the fully edited VGV isoform also display anxiogenic and aggressive behaviors, with altered 5-HTR_{2C} receptor signaling within discrete brain regions in these mice (35). Thus, editing at this locus may mediate altered $5HTR_{2C}$ signaling within the BLA of susceptible mice following CSDS, yet further investigation is needed to assess the functional consequences of CSDS-induced editing at the *Htr2c* locus in this context.

Editing within transcripts encoding Gamma-Aminobutyric Acid Type A ($GABA_A$) and AMPA receptor subunits were also affected following CSDS. Differential editing of sites in the *Gabra3* and *Gria4* transcripts were identified within the BLA 2 and 8 days following CSDS, respectively. The *Gabra3* transcript encodes the α3 $GABA_A$ receptor subunit with editing at this highly conserved site resulting in an isoleucine-to-methionine change in the third transmembrane domain. This site is developmentally regulated and mediates receptor trafficking and GABA sensitivity whereby increased editing is thought to decrease $GABA_A$ receptor signaling (36, 37). Editing at this site was also increased following chronic mild stress within the PFC of adult female rats, suggesting that

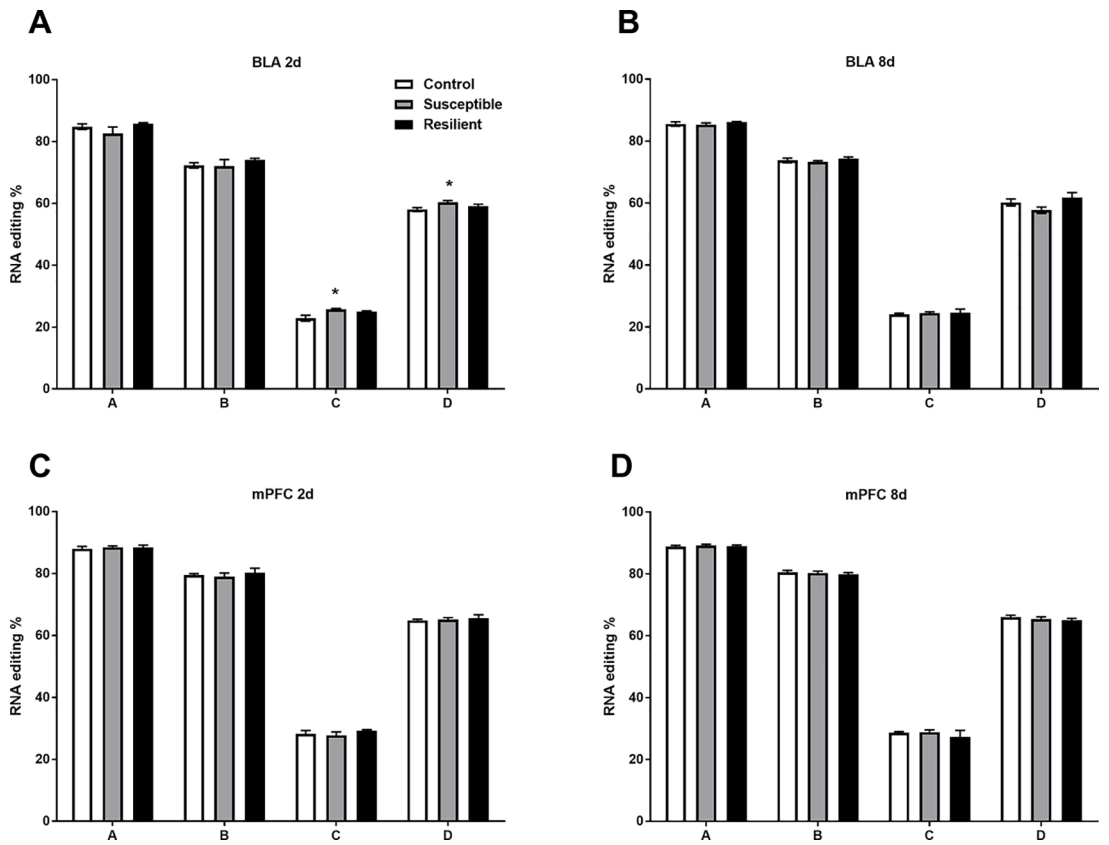

FIGURE 4 | RNA editing of the *Htr2c* transcript is altered within the BLA of susceptible mice 2 days following CSDS. Sequencing analysis revealed modest increases of RNA editing at *Htr2c* sites C and D within the BLA of susceptible but not resilient mice 2 days following CSDS **(A)** with no other changes observed at 8 days **(B)** or at 2 days **(C)** or 8 days within the mPFC **(D)**. Control 2 days, $n = 6–8$; susceptible 2 days, $n = 10$; resilient 2 days, $n = 4$; control 8 days, $n = 7–8$; susceptible 8 days, $n = 7$; resilient 8 days, $n = 3$. $^*p < 0.05$, one-way ANOVA with Sidak *post hoc* comparisons.

this site may be sensitive to various stress modalities in different contexts (27). Moreover, modulation of $GABA_A$ signaling within the BLA impairs SI in rats (38). Alterations in $GABA_A$ signaling *via* RNA editing may contribute to the social avoidance phenotype observed in susceptible mice in this model, although the functional consequences of stress-induced *Gabra3* editing need to be established following CSDS.

Glutamatergic signaling may also be affected by stress-induced RNA editing as increased editing within the *Gria4* transcript encoding the AMPAR α4 subunit in the BLA of susceptible mice at 8 days is indicative of more persistent changes in the RNA editome. Editing at this *Gria4* site confers differences in AMPA receptorchannel kinetics due to regulation of *Gria4* splicing variants, which is sensitive to neuronal stimulation in rat primary cortical neurons (39). Thus, decreased editing in susceptible mice may mediate AMPAR signaling deficits in the BLA in part *via* RNA editing. Indeed, differential AMPAR signaling has been reported within the PFC (40) and hippocampus (41) following CSDS in mice. Further work is required to assess the role of AMPAR signaling, as well as GABAergic signaling, in stress susceptibility following CSDS.

Apart from changes in the aforementioned established editing sites, we aimed to identify novel editing sites sensitive to stress-induced regulation following CSDS. One such example is the *Nova1* transcript encoding the RBP NOVA1. Editing at this *Nova1* recoding site results in a serine-to-glycine exchange, which stabilizes the NOVA1 protein by decreasing proteasome-mediated degradation (42). Interestingly, we identified modest changes in *Nova1* editing in both the BLA and mPFC 8 days following CSDS with increased and decreased editing observed, respectively. Within the BLA, *Nova1* was similarly edited in both susceptible and resilient mice with differential editing only observed within the mPFC of susceptible mice, suggesting that *Nova1* editing is regulated in a region-specific manner following chronic stress. Considering the effects of RNA editing upon NOVA1 protein stability and the lack of CSDS-induced changes in *Nova1* mRNA levels, it would be of interest to assess NOVA1 protein levels within the BLA and mPFC following CSDS. Moreover, considering the established role of NOVA1 as an important RBP within the brain, which mediates both alternative splicing (43) and miRNA activity (44), NOVA1 is an interesting novel candidate requiring further investigation for its role in stress-induced regulation of the transcriptome.

Apart from such nonsynonymous recoding sites, the majority of differential editing sites identified in the current study were located within the 3′UTR of various transcripts such that the

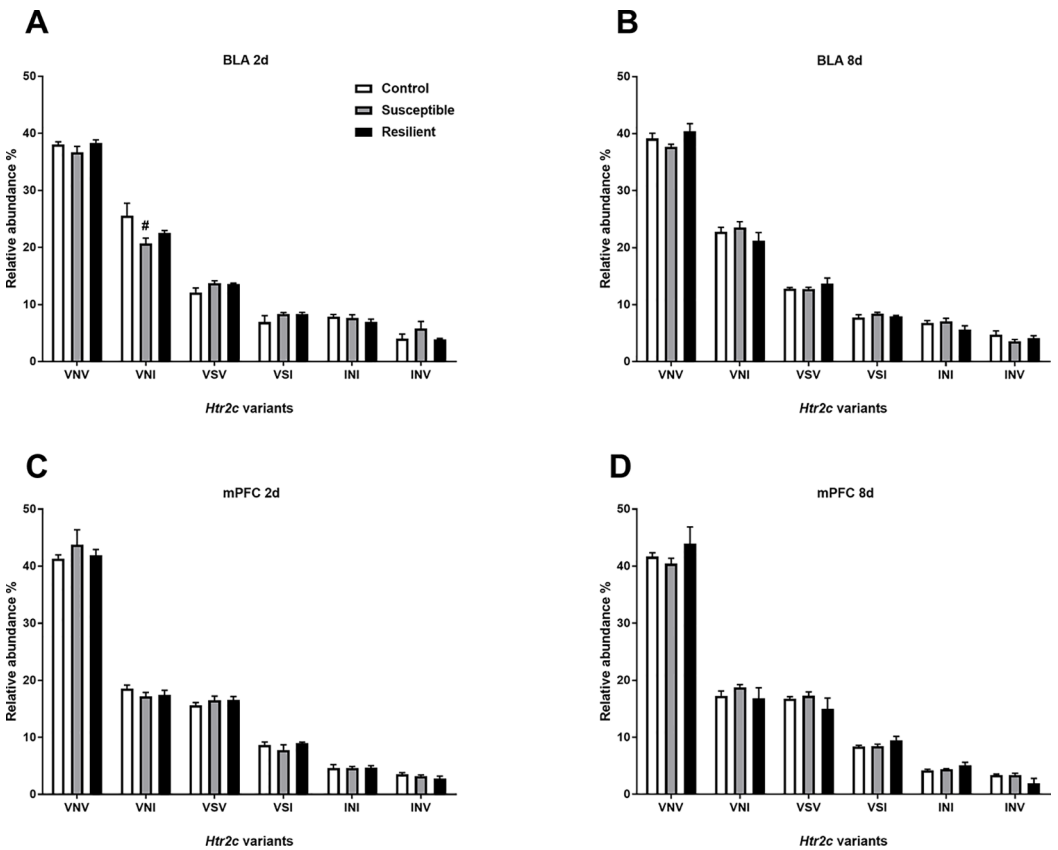

FIGURE 5 | Relative abundance of *Htr2c* variants is minimally altered within the BLA 2 days following CSDS. CSDS induced a nominally significant decrease of the VNI *Htr2c* transcript variant in the BLA of susceptible mice 2 days following CSDS **(A)** with no other changes observed at 8 days **(B)** or at 2 days **(C)** or 8 days within the mPFC **(D)**. Control 2 days, n = 6–8; susceptible 2 days, n = 10; resilient 2 days, n = 4; control 8 days, n = 7–8; susceptible 8 days, n = 7; resilient 8 days, n = 3. $^{\#}p$ = 0.058, one-way ANOVA with Sidak *post hoc* comparisons.

protein coding capacity of these mRNAs remains unaltered. However, RNA editing within 3′UTRs regulates mRNA availability and translation efficiency due to the editing of miRNA binding sites, which can induce differential miRNA-mediated regulation of edited transcripts (6). Persistent increases in editing at a site within the 3′UTR of *Commd2* in the mPFC of susceptible mice did not, however, alter mRNA levels in this brain region, suggesting that miRNA activity at this site is likely unaffected by editing at the site examined in this context. Further studies are required to investigate the consequences of CSDS-induced editing in 3′UTRs in this study including those sites identified in resilient mice such as in the 3′UTR of *Ncl* encoding the eukaryotic nucleolar phosphoprotein Nucelolin, which interestingly interacts with the brain-specific small nucleolar RNA MBII-52, known to regulate *Htr2c* editing within the mammalian brain (45). Furthermore, many differentially edited sites were identified within transcripts encoding proteins with poorly understood functions, particularly in resilient mice (e.g., *Bri3bp* and *Slc35e1*), which should be the focus of further investigation.

Several caveats to this study must be noted, including the small sample size for resilient groups as well as the mainly modest changes in editing levels observed. Repetition of mmPCR-seq analysis in a larger CSDS cohort would likely enable identification of further stress-sensitive editing sites, particularly those associated with resiliency. Moreover, utilizing RNA-seq would enable transcriptome-wide analysis of stress-induced editing and mRNA levels. Despite this, the A-to-I editing changes observed in this study are similar with the editing level changes observed in the rat PFC and amygdala following chronic stress employing a similar mmPCR-seq technique (27). Such modest changes of RNA editing in bulk brain tissue are also likely explained by cellular heterogeneity as recent advances in single-cell transcriptomics have demonstrated that A-to-I editing is indeed cell type specific with changes even observed between different cells of given cellular population within the mammalian brain (46, 47). Thus, it would be of interest in the future to study stress-induced changes in RNA editing using single cell transcriptomics.

In conclusion, the current study has identified A-to-I editing as another molecular mechanism of likely relevance to stress resiliency and susceptibility to CSDS in adult mice, in line with the growing appreciation for stress-induced regulation of RNA metabolism within the brain (21, 48, 49). Further investigation of the consequences of these editing changes is required at both the mRNA and protein levels to decipher the functional consequences of RNA editing following chronic stress.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the European Communities; Council Directive 2010/63/EU. The protocols were approved by the Animal Care and Use Committee of the Government of Upper Bavaria, Munich, Germany.

AUTHOR CONTRIBUTIONS

AD, KK, EL, and AC conceptualized and designed the experiments and wrote the manuscript. AD and EP conducted all animal experiments analyzed by AD. AD and KK conducted and analyzed all mmPCR-seq experiments. AD and FS conducted qPCR experiments analyzed by AD.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00277/full#supplementary-material

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MINI REVIEW
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Is There a Role of Autophagy in Depression and Antidepressant Action?

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Autophagy has been recognized as evolutionary conserved intracellular pathway that ensures energy, organelle, and protein homeostasis through lysosomal degradation of damaged macromolecules and organelles. It is activated under various stress situations, e.g., food deprivation or proteotoxic conditions. Autophagy has been linked to several diseases, more recently also including stress-related diseases such as depression. A growing number of publications report on the role of autophagy in neurons, also referred to as "neuronal autophagy" on the one hand, and several studies describe effects of antidepressants—or of compounds that exert antidepressant-like actions—on autophagy on the other hand. This minireview highlights the emerging evidence for the involvement of autophagy in the pathology and treatment of depression and discusses current limitations as well as potential avenues for future research.

Keywords: autophagy, depression, antidepressant, stress, FKBP51 signalling

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DEPRESSION IS A PREVALENT AND SEVERE DISEASE

Worldwide, depression is one of the most frequent clinical conditions and the leading cause of disability affecting more than 300 million people of all ages, according to World Health Organization (WHO) statistics (http://www.who.int/news-room/fact-sheets/detail/depression). Depression is characterized by a cluster of symptoms that include depressed mood, fear, feelings of worthlessness, loss of energy and interest, reduced responsiveness to pleasurable stimuli, lack of appetite, cognitive impairment, and sleep disturbances (1). A high percentage of seriously depressed patients receive no appropriate treatment, even in developed countries (2). Suicidal ideation is a further characteristic of depression and up to 15% of severely depressed individuals commit suicide. Depression represents also a major independent risk factor for other diseases like cardiovascular disease, dementia, diabetes, and osteoporosis (3, 4).

The high complexity of this mental disorder accounts for the difficulties in elucidating its molecular underpinnings. Overall, it has been increasingly accepted that a multitude of factors ranging from genetic predisposition to environmental challenges contribute to the pathophysiology of depression. In addition to the analysis of specific targets, research efforts increasingly resort to screening platforms to probe the genome, epigenome, etc. in an unbiased way. Examples of the major specific systems under investigation are monoaminergic, glutamatergic, and stress hormone systems, neuropeptides as modulators of the neuronal cell function including neurogenesis, neuronal morphology, and intracellular signaling pathways.

In genetics, huge efforts produced intriguing results; however, the field is haunted by the lack of consistency and reproducibility [for a recent review, see Ref. (5)]. Thus, increasingly large cohorts are investigated, and meta-analyses are employed to probe several hundred thousands of individuals (6).

Nevertheless, not the least due to the difficulties to move from gene association to molecular mechanism, hypothesis-driven approaches continue to be pursued intensely.

Monoamine deficiency was the first hypothesis unfolded over several years, tracing back more than half a century, and probably is the most influential one (7, 8). It postulates lack of monoaminergic neurotransmitters and thus impaired synaptic neurotransmission as cause for depression; several newer antidepressant drugs were developed based on this hypothesis. Other examples include glutamatergic dysfunction and the corticosteroid hypothesis of depression (7, 9). A vast array of studies supports the link between the stress hormone system and depression (9, 10). More specifically, impaired corticosteroid receptor function has been suggested to result in inappropriately high secretions of corticotropin releasing hormone (CRH), vasopressin, adrenocorticotropin, and cortisol (9). A role of autophagy in depression is a more recent hypothesis put forward (11), which can be viewed as one of the ramifications of the stress response as outlined below.

AUTOPHAGY IS A CELLULAR HOMEOSTASIS PROCESS AND PART OF THE STRESS RESPONSE

Autophagy is a pivotal process to ensure homeostasis of cells, and thus of tissues and the organism, in physiological as well as pathological situations (12, 13). This highly conserved mechanism leads to the degradation of damaged cytosolic proteins, aggregates, organelles, and also pathogens through a step-wise process. The basic mechanism is detailed in several excellent reviews (13–15), so it is described here only briefly: Autophagy involves a series of autophagy-related genes (ATGs), originally identified in yeast. Initially, membrane material is excised, most likely from the endoplasmic reticulum, giving rise to a membrane sac that is further expanded to form a double membrane vesicle called autophagosome. To be degraded material is enclosed into this vesicle; selected additional material can be transferred into the autophagosome. Degradation is achieved upon fusion with lysosomes to form autolysosomes: From the initial isolation of membrane material needed for the formation of autophagosomes to the final fusion step, autophagy involves a number of proteins governing membrane dynamics (16). There are different types of autophagy, with macroautophagy being the most commonly described one (15); this review only deals with macroautophagy, because research on the emerging subject of neuronal autophagy did not yet aim at specifying the type of autophagy.

The crucial physiological role of autophagy is reflected in its links to several diseases and the increasing efforts to exploit this process for pharmacological intervention (17–21). Initially, autophagy was identified as response to calorie restriction to maintain energy homeostasis (22). Today, several pharmacological and environmental factors are known to induce autophagy, in particular various kinds of stressors (13, 17). Thus, autophagy is an important facet of the stress response, and like the stress response in general, autophagy is a beneficial process, but excess activation can be detrimental under certain conditions (23, 24). For example, apoptosis (often referred to as Type I cell death) and autophagy are considered mutually exclusive (13). Others debate this exclusiveness and argue that excessive autophagy can cause type II cell death characterized by the formation of large autophagic vacuoles (25, 26).

Chronic stress in mice, which frequently is used to model depression (27, 28), also has been reported to enhance autophagy [for recent examples, see Refs. (29, 30)]. The observation that a further increase in autophagic markers goes along with the reversal of the behavioral effects again argues in favor of autophagy being a beneficial component of the stress response in general (13, 30). Nevertheless, evidence also has been provided for a role of autophagy induction for depressive-like behavior and cognitive impairment induced by prenatal stress (31). Very recently, inhibition of autophagy was shown to attenuate the induction of depressive-like behavior by ecstasy in rats (32).

AUTOPHAGY IN DEPRESSION: EVIDENCE FROM DISEASE AND DISEASE MODEL STUDIES

In human, a study using a small sample size found elevated expression of autophagy genes in blood mononuclear cells from individuals suffering from major depression in comparison to healthy controls (33). Similarly, decreased mRNA expression of AKT1 and mTOR was found in individuals with short-term bipolar disorder compared to healthy controls (34), which might lead to the induction of autophagy. Similarly, a post-mortem study revealed compromised mTOR signaling in the prefrontal cortex in major depressive disorder (35). How could this be reconciled with the observation that enhanced autophagy response in blood mononuclear cells to *ex vivo* antidepressant treatment predicts clinical treatment success (36)? Similar to the stress response in general, autophagy is a beneficial response up to a certain limit, so we hypothesize that this adaptation might be insufficient in some (disease) cases and needs further boosting through various kinds of treatments.

Short-term calorie restriction, one of the most efficient inducers of autophagy (22), has been reported to have antidepressant effects in human and antidepressant-like effects in mice, while the effects of long-term calorie restriction are controversial (37). Likewise, physical exercise has been shown both to enhance autophagy (38) and to reduce depressive symptoms in human (39). Nevertheless, given the plethora of effects of both calorie restriction and exercise, these studies only provide a rather vague support of a potential link between autophagy and depression.

Studies more directly documenting a link of autophagy to psychiatric disease mainly were performed with animal models, with all the debated limitations that come with animal models that try to replicate aspects of depression (27). Maternal separation (40) increased autophagic markers in the prefrontal cortex, but not in the hippocampus (41). This is mimicked by the differential effect of corticosterone in primary astrocytes from these brain regions (42), while another study found that prenatal stress significantly elevated autophagy markers in the hippocampus of male offspring (31). On the other hand, signs of decreased

autophagy also have been reported in depression-relevant animal models. For example, chronic unpredictable stress decreased autophagic markers (43, 44). LPS as well as unpredictable chronic mild stress induced depression-like symptoms in rodents along with reduced expression of autophagic markers (45, 46). Furthermore, inhibition of the autophagy initiator Beclin1 (47) induced depression-like behavioral changes in mice (48). Thus, no consistent picture of enhanced or reduced autophagy in depression yet emerges from animal models. Further, it is difficult to conclude about functional autophagy, as flux assays or determining turnover of long-lived proteins is complicated to perform in mice.

AUTOPHAGY IN DEPRESSION: EVIDENCE FROM TREATMENT EFFECTS

Given the scarcity of studies on disease correlation, the hypothesis that autophagy is involved in depression mainly is based on the effects of antidepressants on autophagy. One of the earliest hints for a role of antidepressants in autophagy was the observation of autophagy-associated structures in the cytoplasm upon treatment of cells with the tricyclic antidepressant clomipramine (chlorimipramine) (49). This phenomenon could be caused by either induction of autophagy or blocking the autophagy flux, thus actually blocking functional autophagy. It should be noted here that the conclusion of active autophagy often is based on the mere appearance of autophagic markers, which is not correct in the absence of experiments assessing the autophagic flux or turnover of long-lived proteins (50). Employing appropriate experiments, it was shown later that desmethylclomipramine, the active metabolite of clomipramine, interferes with the autophagic flux and thus functional autophagy (51). In contrast to the effect of clomipramine, another tricyclic antidepressant, amitriptyline, was found to increase autophagy in primary neurons and astrocytes, similarly to the selective serotonin reuptake inhibitor citalopram; however, the selective serotonin and noradrenaline reuptake inhibitor venlafaxine did not alter autophagy (52, 53). Thus, it appears that antidepressants diversely impact functional autophagy, possibly also in a cell-type-dependent manner.

Conspicuously, the canonical autophagy inducer rapamycin has been found to exert antidepressant-like effects (54, 55), emphasizing the role of the mTOR pathway (56). Conversely, several other established antidepressants and compounds that are reported to exert antidepressant-like effects were shown to modulate autophagy in various experimental models. Among the established antidepressants are the tricyclic antidepressants desipramine, nortriptyline, and imipramine, the tetracyclic antidepressants maprotiline and mianserin, the noradrenergic and serotonergic antidepressant mirtazapine, the selective serotonin reuptake inhibitors fluoxetine (Prozac), sertraline, and paroxetine, the serotonin-norepinephrine reuptake inhibitor desvenlafaxine, the atypical antidepressant agomelatine, lithium [for a review, see Ref. (57)], and the anticonvulsant valproic acid. Further drugs with both antidepressant-like effects and impact on autophagy include trehalose, hypericin, which is one of the principal components of Saint John's wort, Salvianolic acid B, rosiglitazone, silibinin, dapsone, geldanamycin, α-tocopherol, and extracts of Euryale ferox Salisb (see **Table 1** for more details and citations). Of note, also electroconvulsive therapy, which particularly is used for severe or treatment-resistant depression (58), was reported to enhance autophagy (59).

Mechanistically, antidepressants appear to address various pathways to impact autophagy. For example, FKBP51, which is a glucocorticoid receptor and stress regulator linked to psychiatric diseases (84–86), has been shown to be required for the effects of antidepressants on both autophagy and depressive-like behavior (36, 87). Another very recent study discovered that the previously reported effects of antidepressants on the acid sphingomyelinase (ASM) (88, 89) trigger a pathway leading to upregulation of autophagy, which is required for the behavioral effects in mice (48). More specifically, this pathway involves the accumulation of antidepressants in lysosomes, where they inhibit ASM. This leads to an increase in sphingomyelin and finally of ceramide in the endoplasmic reticulum. Ceramide, in turn, activates the phosphatase PP2A, which stimulates the kinase ULK, a known activator of autophagy (48).

Of the pleiotropic effects of the mood stabilizer lithium (90), its autophagy-inducing action does not operate through GSK3β, but by inhibition of inositol monophosphatase (91). Despite first glimpses, overall there is considerable lack of mechanistic understanding of how antidepressants link to autophagy. This is partly due to the incomplete knowledge about the molecular interaction partners of antidepressants. Progress in this direction (92) will help elucidating the molecular connection to autophagy. This may also contribute to sorting the actions of antidepressants, because not everything antidepressants do has to be related to depression treatment. Another long-standing conundrum in understanding how antidepressants work is the observation that clinical effects typically take weeks to become manifest, while known targets like neurotransmitter transporters are affected immediately. It is unlikely that autophagy will offer an obvious solution. Arguably, it contributes to starting a process of neuronal reorganization that ultimately constitutes the transition from disease to health (cf. **Figure 1**). Neurogenesis might be part of this process, as extensively discussed elsewhere (93). In this context, it is intriguing that autophagy increases adult neurogenesis (94, 95); thus, it is possible that antidepressants and lithium operate, at least in part, through autophagy to induce neurogenesis (90, 96).

The vast majority of publications report an increase in autophagy by antidepressants. This is also the case for the fast-acting antidepressant ketamine (75), even though it is known to enhance mTOR activity (98). However, as alluded to above, flux assays are missing in many studies (cf. **Table 1**), which may lead to erroneous interpretation and conflicting results. More specifically, many of these reports merely observed an upregulation of autophagic markers, for example, lipidation of LC3B (i.e., an increase in the ratio of LC3II/I), which is not sufficient to make conclusions about functional autophagy. Overall it appears more likely that antidepressants diversely affect autophagy. Another important issue is the concentration at which antidepressants are administered in experimental models. This concentration typically is in the range of 10 μM n cell culture or 10 mg/kg in

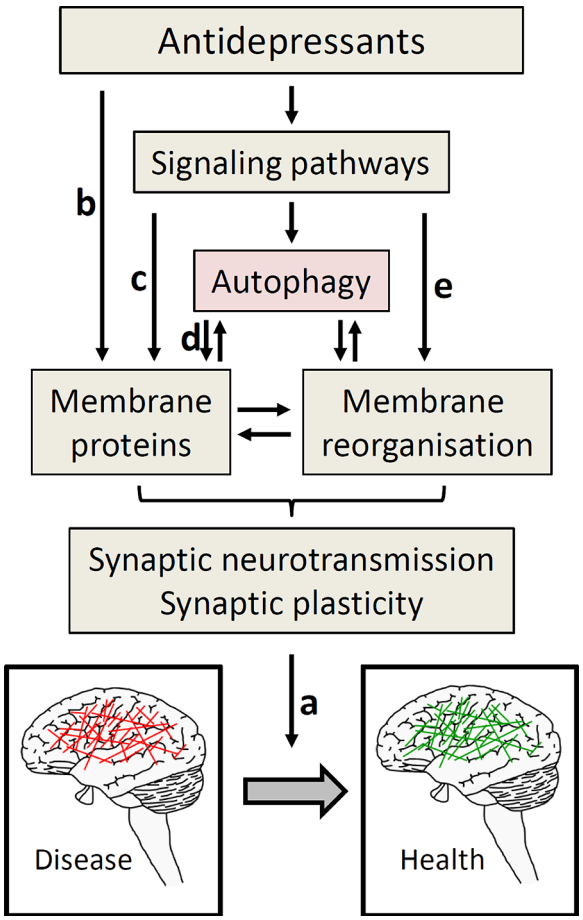

FIGURE 1 | Autophagy as part of antidepressant action. To move from diseased (depressed) to healthy state, ultimately a change in neuronal activity is required (A). To achieve this, several ways of antidepressant actions are proposed including effects on hormonal systems, immune system, and neurogenesis, which all might be intertwined with autophagy (97); this figure focuses on synaptic neurotransmission. The by far most often described effect of antidepressants on synaptic neurotransmission operates through directly blocking neurotransmitter reuptake transporters (part of the membrane proteins, (B). These transporters may also be addressed through signaling pathways that regulate their expression and/or function (C), not part of this review). The role of autophagy in antidepressant action frequently is explained by maintaining protein homeostasis in general, and the functional integrity of membrane proteins involved in synaptic neurotransmission in particular (D). These membrane proteins comprise not only transport proteins, but also, e.g., presynaptic SNARE proteins engaged in neurotransmission. Given the similarity of membrane dynamic processes in autophagy and synaptic neurotransmission, and to reconcile the diverse findings of antidepressant effects on autophagy, we also discussed the hypothesis that antidepressants address pathways that change membrane organization, directly linking to synaptic neurotransmission (E).

animal experiments, sometimes even higher. While it has been reported that similar doses can be reached in the brain (99, 100), effects reached at concentrations based on the results of therapeutic drug monitoring (101) may more closely mimic the clinical situation. For example, paroxetine (used at the therapeutic drug dose of 120 ng/ml = 0.9 μM) and amitriptyline (used at the therapeutic drug dose of 120 mg/ml = 0.37 μM) enhanced the expression of autophagy markers in blood mononuclear cells from depressed patients exposed to these reduced concentrations *ex vivo* (36).

CONCLUSION AND OUTLOOK

Over the last few years, several studies provided evidence for a link of autophagy to the pathophysiology and treatment of depression. Despite impressive progress, the mechanism is far from being understood. This is not surprising for a complex disease like depression, which poses particular experimental challenges and epistemological limitations, as exemplified by the complex mechanisms linking stress with depressive behavior. The molecular effects of antidepressants ultimately need to produce alterations in the pattern of neuronal activity that underlie the transition between diseased and healthy status (**Figure 1**). This means that some neuronal activity needs to be decreased and some needs to be increased. Interestingly, neuronal stimulation not only induces autophagy (102); increased autophagy also impacts synaptic function. For example, induction of autophagy by mTOR inhibition in presynaptic terminals rapidly alters presynaptic structure and reduces neurotransmission (103). Conversely, loss of autophagy slows down synaptic neurotransmission while gain of autophagy increases it (104). The latter finding has been conceptualized by the function of autophagy in protein homeostasis by removing damaged proteins, in this case those involved in synaptic vesicle exocytosis in particular (104, 105). Intriguingly in a mouse model of learnt helplessness evoking depressive-like behavior, decreased levels of the presynaptic vesicle membrane docking and fusion SNARE protein Snap25a occur along with impaired autophagy; administration of fluoxetine attenuates both these effects (106). The SNARE proteins are important components of the membrane reorganizing machinery at the synaptic membrane, and there is an interdependence between autophagy and synaptic vesicle trafficking (107, 108). In addition, electroconvulsive therapy enhances not only autophagic markers (59) but also the membrane trafficking machinery (109).

In light of the presumably diverse impact of antidepressants on autophagy, enhanced recycling of distinct synaptic proteins by inducing autophagy is unlikely to fully picture the mechanism of antidepressants. Given the fact that autophagy needs the activity of a number of membrane reorganizing and membrane trafficking proteins, we consider it plausible that processes impacting autophagy may also impact membrane reorganizing processes at the synapse, and thus would not require the later steps autophagy (cf. **Figure 1**). In general, these processes could be fast, because they do not necessarily require the synthesis of new proteins. They could limit synaptic neurotransmission when autophagy and neurotransmission compete for additional membrane material; conversely, autophagy would promote neurotransmission if there are shared mechanisms for the generation and fusion of membrane material. Thus, it will be of great interest to learn about the conditions under which autophagy increases or decreases synaptic neurotransmission, possibly in a neurotransmitter-specific fashion.

TABLE 1 | Overview of the various autophagy-impacting compounds that are used as antidepressants or reported to exert antidepressant-like effects in animal models.

Compound/ Antidepressant	Experimental system	Results, autophagic markers	Flux, LLP	Citation
Clomipramine*,	Human glioma cells	Autophagy-associated structures	no	(49)
Desmethyl-clomipramine	HeLa Cells, ATG5$^{-/-}$MEFs	LC3BII/I up, increase in DM structures, flux blocked, LLP degradation down	yes	(51)
Amitriptyline*	Primary rat astrocytes and neurons, ATG5$^{-/-}$MEFs	Increased autophagy (LC3BII/I, Beclin1 up)	yes	(52)
	Mouse stress model, patient blood cells, HEK cells, rat cortical astrocytes	ATG12, LC3II/I, Beclin1, pAkt1 and VPS34 were up, increased flux	yes	(36)
	Corticosterone-stressed mice	Increased autolysosomes, affects pBeclin, pULK, increased p62	no	(48)
Citalopram*	Primary rat astrocytes and neurons	Increased LC3BII/I and Beclin1	no	(52)
Venlafaxine*	Primary rat astrocytes and neurons	No effect	no	(52)
Desipramine*	C6 glioma cells	Inhibition of mTor pathway, increased Beclin1, LC3, autophagosomes	no	(60)
	L929 cells ATG7$^{-/-}$ MEFs	Autophagy induction (LC3II/I up, p62 down,	no	(61)
Nortriptyline*	High content chemical screen in HeLa cells	Autophagy induction (LC3II/I, flux)	yes	(62)
Imipramine*	Glioma cells, mouse models of gliomagenesis	Upregulation of LC3II/I, increased flux, more autophagic vacuoles	Yes (cells)	(63)
	THP-1 cells, depressed patients, ATG5$^{-/-}$MEFs	mRNA of LC3 and Beclin1 up, LC3II/I up	no	(64)
	U-87MG glioma cells	Inhibition of PI3K/Akt/mTOR signaling, LC3II/I up	no	(65)
Maprotiline*	Burkitt's lymphoma cell line	Beclin1 up, more cytoplasmic vacuoles	no	(66)
Mianserin*	THP-1 cells, depressed patients	mRNA of LC3 and Beclin1 up	no	(64)
Mirtazapine*	THP-1 cells, depressed patients, ATG5$^{-/-}$MEFs	mRNA of LC3 and Beclin1 up, LC3II/I up	no	(64)
Fluoxetine*	Human breast cancer cell lines	Upregulation of LC3II/I, Beclin1, ATG5; p62 down	yes	(67)
	Human adipose-derived stem cells, mature adipocytes	Upregulation of LC3II/I, ATG12, SQSTM1, Beclin1, ATG7	no	(68)
	Brain injury in rats	Upregulation of Beclin1, LC3 punctae	no	(69)
	Stress model in rats	Upregulation of Beclin1 and LC3II increased PI3K/Akt/mTOR activity.	no	(43)
	Burkitt's lymphoma cell line	Beclin1 up, more cytoplasmic vacuoles	no	(66)
Sertraline*	Non–small cell lung cancer cells	LC3II up, increased flux, autolysosome formation	yes	(70)
	AML cell lines	LC3II/I increased	no	(71)
Paroxetine*	THP-1 cells, depressed patients	mRNA of LC3 and Beclin1 up	no	(64)
	Mouse stress model, patient blood cells, HEK cells, rat cortical astrocytes	ATG12, LC3II/I, Beclin1, pAkt1 and VPs34 were up, increased flux	yes	(36)
Desvenlafaxine*	THP-1 cells, depressed patients	mRNA of LC3 and Beclin1 up	no	(64)
Agomelatine#	THP-1 cells, depressed patients	mRNA of LC3 and Beclin1 up	no	(64)
Lithium*	ALS mouse model	Increased number of autophagic vacuoles (Beclin1 and LC3)	no	(72)
	Prion-infected cells	LC3II/I and flux increased	yes	(73)
VPA*	Human glioma cell lines	LC3II/I and Beclin1 increased	no	(74)
Ketamine*	Human epithelial cells	LC3II/I and Beclin1 increased	no	(75)
Trehalose	Mouse model of manic-like behaviors	Reduced ratio of p62/beclin1 in the frontal cortex	no	(76)
	Diverse mammalian cells, ATG5$^{-/-}$MEFs	Increased LC3II/I, flux	yes	(77)
Hypericin	Human macrophages	LC3II/I and Beclin1 up, p62 down, only in combination with ultrasound	no	(78)
	Leishmania promastigotes	mRNA of AMPK up, ATGs diversely regulated	no	(79)
Salvianolic acid B	Depression model in rats	Compound restores treatment-induced impairment of autophagy (LC3II/I, Beclin1)	no	(46)
Rosiglitazone*	Depression mouse model, N2a cells, primary neurons	Increases Beclin1, ULK1, LC3II/I, pAMPK, and pAKT1, decreases p62 in stressed mice	no	(45)
Silibinin#	Depression mouse model	Decreased LC3II/I	no	(80)
Dapsone*	Cognition-compromised rats	Enhanced LC3II/I and Beclin1, decreased p62	no	(81)
Geldanamycin	Rat model of anxiety and depression	Atg12, Atg7, and LC3II/I increased	no	(82)
α-tocopherol*	Mouse model of depression	Enhanced LC3II/I, pAMPK decreased p62, pmTOR	no	(44)
Euryale ferox Salisb extracts	Mouse model of depression, HT22 cells	Enhanced LC3II/I, pAMPK decreased p62, pmTOR	no	(83)

**Labels drugs approved by the United States Food and Drug Administration. #Approved in the European Union. LLP, assay to determine the stability of long-lived proteins; DM, double membrane; MEF, mouse embryonic fibroblasts; VPA, valproic acid; AML, acute myeloid leukemia; ALS, amyotrophic lateral sclerosis.*

Experiments employing genetic and pharmacological intervention strategies are needed to finally proof the involvement of functional autophagy in antidepressant action and to disentangle the mechanism including the level of synaptic neurotransmission. More specifically, (conditional) knock-outs of central autophagy genes are available in mice. The high interest in autophagy modulators has led to the discovery of a range of novel autophagy inducers and inhibitors, which can be tested in animal models in depression. While compounds that inhibit autophagy through blocking the fusion between autophagosome

and lysosome frequently elicit toxic effects when applied over a long period of time, they might be useful for assessing the role of autophagy in the immediate actions of antidepressants in some test regimes such as the forced swim test. It will also be interesting to learn whether and how antidepressants can be grouped according to their impact on autophagy. This categorization may not follow the pattern of their mechanism so far known. Finally, it should be investigated whether the dose dependency for autophagy induction by antidepressants is the same as or at least similar to the therapeutic doses.

AUTHOR CONTRIBUTIONS

TR drafted the manuscript. NG added critical information.

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REVIEW
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A Major Role for the Lateral Habenula in Depressive Illness: Physiologic and Molecular Mechanisms

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Emerging preclinical and clinical evidence indicate that the lateral habenula plays a major role in the pathophysiology of depressive illness. Aberrant increases in neuronal activity in the lateral habenula, an anti-reward center, signals down-regulation of brainstem dopaminergic and serotonergic firing, leading to anhedonia, helplessness, excessive focus on negative experiences, and, hence, depressive symptomatology. The lateral habenula has distinctive regulatory adaptive role to stress regulation in part due to its bidirectional connectivity with the hypothalamic–pituitary–adrenal (HPA) axis. In addition, studies show that increased lateral habenula activity affects components of sleep regulation including slow wave activity and rapid eye movement (REM), both disrupted in depressive illness. Lack of perceived reward experienced during the adverse outcomes also precipitates lateral habenula firing, while outcomes that meet or exceed expectations decrease lateral habenula firing and, in turn, increase midbrain dopaminergic and serotonergic neurotransmission. The ability to update expectations of the environment based on rewards and aversive stimuli reflects a potentially important survival mechanism relevant to the capacity to adapt to changing circumstances. What if one lives in a continuously aversive and invalidating environment or under the conditions of chronic stress? If there is a propensity of the habenula to release many burst discharges over time, an individual could habitually come to perceive the world as perpetually disappointing. Conceivably, the lateral habenula could learn to expect an adverse outcome systematically and communicate it more easily. Thus, if the lateral habenula fires more frequently, it may lead to a state of continuous disappointment and hopelessness, akin to depression. Furthermore, postmortem studies reveal that the size of the lateral habenula and total number of neurons are decreased in patients who had depressive illness. Novel research in the field shows that ketamine induces rapid and sustained antidepressant effect. Intriguingly, recent preclinical animal models show that ketamine abolishes *N*-methyl-*D*-aspartate receptor (NMDAR)-dependent lateral habenula bursting activity, leading to rapid resolution of depressive symptoms.

Keywords: ketamine, major depressive disorder, lateral habenula, HPA axis, NMDA-type receptors, sleep

INTRODUCTION

The habenula is a component of the diencephalon and, together with the pineal gland, makes up the epithalamus (1). This evolutionary well-conserved structure plays a central role connecting forebrain and midbrain (2). It is involved in multiple processes that are core components of the major depressive syndrome such as reward processing, cognition, stress adaptation, sleep and circardian rythm regulation, biological rhythms, and the regulation of monoaminergic (dopamine and serotonin) neurotransmission (2–6). Its dysfunction has been implicated in psychiatric illnesses closely related to maladaptive processing of positive and negative valence (7). Further, lateral habenula serves as an interface among emotions, stressors, and cognitions. The hypothalamic–pituitary–adrenal (HPA) axis and lateral habenula appears to have unique bidirectional regulation, thus perturbations of the HPA axis are associated with alterations of lateral habenula function (5).

Lateral Habenula and Reward Processing in Depression: Interaction With Dopaminergic and Serotoninergic Neurotransmission

One of the functions of the lateral habenula is to encode negative motivational values associated with primary punishment in humans (8) and primates (9, 10). Thus, the habenula encodes the values of cues previously paired with an aversive outcome. Accordingly, habenula responses predict the extent to which individuals withdraw or approach negative and positive cues, respectively, thus playing a central role in driving aversive motivated learning and behavior (7, 11). This is accomplished through the extensive connections of the lateral habenula neurons (largely glutamatergic) to dopamine neurons in the ventral tegmental area (VTA) and the substantia nigra (8, 9). In addition, the rostromedial tegmental nucleus (RMTg) also known as the GABAergic tail of the VTA, receives glutamatergic inputs from the lateral habenula and sends substantial GABAergic projections to the midbrain dopaminergic system including VTA, as well dorsal raphe nucleus, locus coeruleus and other regions. Notably, VTA receives direct projections from the lateral habenula as well (6, 7, 9).

When a reward is smaller than expected or the anticipated reward is unsatisfactory, the firing rate of the lateral habenula increases, leading to inhibition of dopamine release from midbrain dopaminergic neurons that project to the nucleus accumbens, highly involved in reward processing (2, 7, 12, 13). Therefore, the lateral habenula is implicated in encoding information about aversive signals or missing rewards. Its firing rate increases in response to chronic stress, punishment, and stimuli that have been previously associated with negatively charged experiences (7, 11). Accordingly, the lateral habenula plays a key function in learning from painful experiences and in making decisions to avoid such aversive experiences in the future (12, 13). In contrast, if the expected reward meets or exceeds our expectations, the firing rate of the lateral habenula decreases, leading to activation of brainstem dopaminergic nuclei, which activate the nucleus accumbens (2, 12–14) that is critically important for mediating and experiencing reward. This activity is thought to help us remember the details of how we obtained the reward. Thus, this will also help us to remember how to get the reward in the future (7). When the encoding of the reward becomes hyperactive, it can result in obsessive reward-seeking behaviors involved in addiction disorders (15).

Lateral habenula also interacts with the raphe nuclei and the serotonergic system. When the firing rate of the lateral habenula is high, the release of serotonin from the raphe nuclei is reduced, resulting in decreased serotonin neurotransmission. Input to the lateral habenula from the basal ganglia increases the firing rate of the lateral habenula, leading to aversive outcomes, but this pathway is suppressed by serotonin (16, 17).

The ability to update expectations of the environment based on rewards and aversive stimuli reflects a potentially important survival mechanism relevant to the capacity to adapt to changing circumstances (12, 13). Theoretically, if one lives in a continuously highly aversive and invalidating environment or under the conditions of chronic stress, there will be a propensity of the habenula to release many burst discharges over time, so that an individual could systematically come to perceive the world as perpetually disappointing (12, 13, 18). Conceivably, the habenula could learn to expect an adverse outcome systematically and communicate it more easily. Thus, Kaye et al. note that if the habenula fires more frequently, it may lead to a state of continuous disappointment and hopelessness, akin to depression. In addition, lateral habenula neuronal activity is significantly enhanced in rodent animal models of depression (13, 19) as well as in depressed patients (20–22).

Given its unique capability to relay information from limbic forebrain to midbrain monoamine nuclei *via* high-density afferents to monoaminergic centers, the lateral habenula could potentially induce the down-regulation of the serotonergic, noradrenergic, and dopaminergic systems. This complex process, resulting from functional hyperactivation of the lateral habenula, has critical implications for regulating aversive behaviors and depressive pathophysiology (2, 16, 23, 24).

Bidirectional Relationship Between Activation of the Hypothalamic–Pituitary–Adrenal Axis and the Lateral Habenula

The paraventricular nucleus of the hypothalamus contains abundant corticotropin-releasing hormone (CRH) neurons, which release CRH to activate the pituitary–adrenal axis. The paraventricular nucleus of the hypothalamus sends direct projections to the lateral habenula, but the functional consequences of this projection are unknown (3, 23). During increased stress and adverse experiences, the HPA axis and the lateral habenula are concomitantly activated (25), but it is not clear, however, which occurs first.

Direct activation of the lateral habenula is associated with HPA axis activation (25, 26). As a corollary, bilateral lesioning of the lateral habenula abolishes the HPA axis and behavioral responses to stress (27). The extent to which these phenomena reflect direct interactions between the lateral habenula and the hypothalamic CRH neurons or proceed by intermediary pathways ultimately linking the habenula to hypothalamic CRH neurons and *vice versa* is currently unknown.

The lateral habenula also expresses CRH receptor 1 (CRHR1) receptors, which are activated *via* restraint stress. CRH activates the lateral habenula (23). Although this stress-mediated activation of habenula CRH receptors is likely to participate in the stress response, this premise remains to be validated. To this end, work from Authement et al., show that CRH-mediated physiological stimulation in slices or behavioral maternal deprivation in rodent pups decreased the abundance of potassium channels in pups, which in turn increases the firing rate of lateral habenula neurons (23). Further work is required to elucidate the relationship among CNS pathways that mediate the relationship between hypothalamic and extrahypothalamic CRH neurons and their consequent behavioral and physiological effects.

Impact of Lateral Habenula on Sleep Alterations in Depression

Sleep is another domain that is systematically disrupted in patients with depressive illness. Most notably, patients with depressive illness have decreased slow wave sleep activity and a systematic increase in rapid eye movement (REM) sleep, as well as a faster onset of REM sleep than controls (28). Specifically, the lateral habenula appears to have a critical role in regulating oscillatory theta hippocampal activity through modulation of temporal dopaminergic and serotoninergic firing pattern, suggesting a synaptic mechanism for memory consolidation during REM sleep, a decrease in slow wave sleep, and a highly significant increase in REM sleep (16, 28). Thus, an increase in the activity of the lateral habenula would result in the decrease in slow wave sleep and the significant augmentation of REM sleep, well-known characteristics of depressive illness.

Glutamatergic Modulation and the Lateral Habenula: Evidence From Preclinical and Clinical Studies

One of the most exciting paradigm shifts in biological psychiatry in the past two decades is the discovery that a single subanesthetic dose of ketamine (a prototypic glutamatergic modulator) to treatment-resistant depressed patients induces rapid and sustained antidepressant responses within hours, often lasting as long as 1–2 weeks (29). This initial hypothesis surrounding the fast-onset antidepressant response was related to direct and indirect *N*-methyl-D-aspartate receptor (NMDAR) inhibition in the hippocampus and medial prefrontal cortex, which in turn induced a rapid increase in neuroplasticity and neurogenesis (30, 31). However, recent animal work regarding the mechanism of ketamine action implicate the conversion of ketamine into an abundant distinct metabolite known as hydroxynorketamine (HNK) found both in human and rodent plasma. HNK seems to be critical in the increase in presynaptic glutamate release, and inducing early and sustained activation of the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR) relative to the NMDAR in hippocampus (32, 33). In fact, Zanos et al., in a series of well-controlled experiments, demonstrated that HNK exerted antidepressant effects on forced swim test and learned helplessness tests independent of NMDAR effects, a process that appears to be related to physiologic activation of AMPAR in the hippocampus and medial prefrontal cortex (32). Blockade of AMPA receptors in these loci with the specific AMPA blocker NBQX abolished the antidepressant effects of ketamine (32). Furthermore, Zanos et al., found that at relevant antidepressant concentrations (10 μM) (2R,6R)-HNK neither inhibited NMDARs nor induced any of the side effects typically associated with ketamine (32, 34). However, at this stage, there are no human studies to substantiate the antidepressant properties of HNK in humans.

Intriguingly, recent work from Yang et al. proposed that inhibition of lateral habenula glutamatergic neurons may be an additional NMDA-dependent mechanism. Their work revealed that local administration of ketamine or other compounds that block NMDAR-inhibited bursting activity of the lateral habenula had a rapid antidepressant effect (35). This group also found that photo-stimulation of lateral habenula drives behavioral despair and anhedonia, which is blocked by inhibiting the NMDA bursting activity effects on monoaminergic reward centers (35). While the blockade of NMDA receptors in the lateral habenula was sufficient to reverse experimentally induced behavioral despair, blockade of low-voltage-sensitive T-type voltage sensitive calcium channels (T-VSCC) was also sufficient to induce rapid-antidepressant effects (35). This work provides a simpler model whereby ketamine quickly (minutes to hours) elevates mood by blocking NMDAR-dependent burst activity of lateral habenula neurons and, in turn, disinhibits downstream monoaminergic reward centers (35). However, it remains unclear whether NMDAR-dependent antagonism alone may be sufficient for ketamine's fast onset and protracted antidepressant effects. While this hypothesis needs to be substantiated in clinical studies, non-NMDAR-mediated glutamatergic potentiation and sustained activation of AMPARs seem to be central to the long-lasting antidepressant effect of ketamine and its main metabolite, HNK.

In an animal model of depression, the learned helplessness test, the lateral habenula activity is significantly increased, and the activity of brainstem dopaminergic neurons was concomitantly inhibited (19). The increase in lateral habenular firing rate resolved with the administration of antidepressants (19). In a congenital animal model of learned helplessness, the lateral habenular firing rate was also increased, which resolved after antidepressant administration (36). In models of depression provoked by maternal separation, lateral habenular firing was increased, but resolved with interventions that normalized the lateral habenular firing rate (37).

Further, a complete pharmacologic stereotaxic inhibition of lateral habenular firing bursts ameliorated depression-like behaviors in rodents and ameliorated the decreased raphe nucleus firing and serotonergic neurotransmission associated with increased habenular neuronal activity (36). The latter finding is compatible with work showing that activation of the lateral habenula inhibits the serotonergic raphe nuclear firing rates. In addition, Yang et al. show that acute ketamine treatment inhibits NMDAR-dependent burst activity in the lateral habenula, resulting in the disinhibition of the downstream activity of midbrain dopaminergic neurons and serotoninergic neurons, which are responsible for activating the reward centers in the brain. As noted, local blockade of NMDARs or low-voltage-sensitive T-type voltage sensitive calcium channels (T-VSCCs) in the lateral habenula sufficed to induce rapid

antidepressant effects (35). Furthermore, in an animal model of maternal-deprivation-induced severe early life stress, a single *in vivo* administration of ketamine induced long-lasting antidepressant effects as well as the reversal of lateral habenula neuronal dysfunction up to 72 h post-injection (38).

Prior research has shown that lateral habenula and the serotoninergic neurotransmission are bidirectionally interconnected, but the functional role of this interconnection has been largely elusive. Recently, scientists have been able to shed light into this functional interconnectivity using optogenetic or pharmacological approaches through perturbation of serotonin signaling, which influences lateral habenula activity. Indeed, tryptophan depletion in patients with depressive illness increases cerebral blood flow in the lateral habenula and initiates increased firing rates, indicated by the lateral habenula's capacity to down-regulate raphe nucleus activity in patients with depressed mood (24). Moreover, Carlson et al. showed that single-dose ketamine infusion in treatment-resistant major depressive disorder (MDD) patients abolishes glucose hypermetabolism in lateral habenula compared to baseline (39), indicative of normalization of habenular brain activity following ketamine infusion.

A postmortem histologic study in MDD patients showed not only decreased size of the lateral habenula but also a reduction in the total number of lateral habenular neurons (40). While limited in number and power, the habenular volumetric alteration studies have produced mixed results for the field so far, in part due to limited spatial resolution and delineation of the structure. For example, a previous study in patients with depressive illness revealed volumetric reduction of the lateral habenula that was most prominent in bipolar depressed patients and in female patients undergoing a current major depressive episode (41). In contrast, Liu et al. show increased habenula volume in unmedicated depressed subjects compared to healthy volunteers (42). Interestingly, the study showed a positive association of habenula volume and more severe anhedonia scores. Consistent with that, other studies have found that greater habenula volume was associated with onset of first-episode depression (43) and correlated with depression symptom severity scores (44).

Deep brain stimulation of the lateral habenula of a patient with severe treatment-resistant depression completely abolished depressive symptomatology. Intriguingly, an inadvertent cessation of stimulation, the subject experienced a relapse of depression but regained remission after the reinstatement of habenular stimulation (22).

Lawson et al. found that, in healthy volunteers, lateral habenula activation increased as conditioned stimuli became more strongly associated with electrically induced shocks. This pattern was significantly different in depressed subjects, for whom habenula activation decreased significantly with increasing association between conditioned stimuli and electric shocks. In both volunteers and patients, individual differences in habenula volume were negatively associated with symptoms of anhedonia (20). In this study, depressed subjects exhibited abnormal negative task-related habenula responses during aversive conditioning. The direction of this effect is opposite to that predicted by other accounts of depression based on findings in animal models. The authors speculate that the negative habenula responses may result by the loss of the capacity to actively avoid negative cues in MDD, which could lead to excessive negative focus (8, 20).

Molecular Mechanisms

While lateral habenula neurons are primarily glutamatergic, its input come from discrete brain areas and are both excitatory (glutamatergic) and inhibitory (GABAergic) in nature (3). These inputs are integrated into complex bidirectional downstream regulatory fashion with the monoaminergic system *via* both direct and indirect connections to the VTA. In addition, the lateral habenula indirectly inhibits dopaminergic neurons in VTA and serotoninergic neurons in raphe nuclei through GABAergic RMTg, conveying information indicative of negative reward and aversive stimuli (15, 45). In animal models, lateral habenular circuits are involved in behavioral avoidance and inhibition of motor response appraised through various afferent circuits and efferent circuit to RMTg (45). Experimental activation of lateral habenular circuits in animal models produces active, passive, and conditioned behavioral avoidance (2). As noted, direct inhibition of lateral habenular NMDA receptors either optogenetically or *via* the use of ketamine produces rapid-acting antidepressant effects in multiple animal models of depression-like syndromes (35).

Cui et al. demonstrated that an astroglial potassium channel Kir4.1 is up-regulated in the lateral habenular model of depression, associated with an increased firing rate of the lateral habenula (46). Loss of Kir4.1 in the lateral habenula ameliorates the increased firing rate of this structure and resolves depressive symptomatology (46). Specific gain of Kir4.1 in the lateral habenula, on the other hand, promotes depressive symptomatology (46). Thus, Kir4.1 in the lateral habenula may serve as a conceivable target for the drug treatment discovery in depression.

The mechanism by which an up-regulated Kir4.1 mediates depression has not been definitively established. Recent data indicate that up-regulated Kir4.1 may lead to neuronal hyperpolarization, inactivating T-type voltage-sensitive calcium channels, which in turn lead to NMDAR bursts that ultimately result in increased suppression of downstream monoaminergic centers. Hence, as noted, ketamine blockage of lateral habenula NMDA receptors results in amelioration of depression-like symptoms in rodents (35).

P11 is another multifunctional protein that interacts with serotonin receptor enzymes, chromatin remodeling factors, and ion channels, which are critically involved in depression-like behaviors and antidepressant actions (18). p11 is enriched in distinct neuronal types, especially in the nucleus accumbens. A previous study revealed that chronic stress leads to an increase in p11 in dopamine D2 neurons, which contributes to behaviors suggestive of depression in an animal model of depression-like behaviors in animal models. P11 is also significantly increased in the lateral habenula of chronicly stressed animal models (18). Specific knockout of p11 in the lateral habenula alleviates the stress-induced depressive

behaviors. On the other hand, overexpression of p11 in the lateral habenula results in depressive behaviors (18). Thus, p11 may appears to have a key role in the pathophysiology of depression and an interesting target for pharmacological intervention.

Using a quantitative proteomic screen, Li et al. discovered a signaling pathway enzyme known as the β form of calcium/calmodulin-dependent protein kinase type II (βCaMKII), which, when overexpressed in the lateral habenula, produced depressive-like behaviors (47). Manipulations increasing the presence of βCaMKII consistently increased depressive behaviors, including anhedonia and behavioral despair (48). In contrast, antidepressant medications and RNA interference of βCaMKII significantly diminished depressive manifestations (48). The mechanisms by which up-regulation of βCaMKII leads to increased firing rates of the lateral habenula have not been definitively determined, yet βCaMKII, nevertheless, represents a potential target for ameliorating depressive symptomatology.

Stressful stimuli that increase lateral habenular firing rates and promote a depressive behavioral phenotype increase protein phosphatase 2A (PP2A), which has known influence on the functional activity of the $GABA_B$ receptor that regulate G-coupled inwardly rectifying potassium channel receptor (GIRK) (49). Specifically, chronic stress causes significant weakening of $GABA_B$ GIRK function and neuronal excitability, which is restored by pharmacologic inhibition of PP2A (49). Thus, PP2A inhibitors may have therapeutic efficacy in depressive syndromes associated with increased firing of lateral habenula neurons. More so, recent data indicate that pharmacological activation $GABA_B$ receptor or axon-sparing lesion in the RMTg, a region that receives dense projection from lateral habenula, significantly suppresses the lateral habenula firing rate (50). Hence, the restoration $GABA_B$ signaling may ameliorate depression symptomatology (49).

In sum, the confluence effect of stress-induced up-regulation of Kir4.1, p11, βCaMKII, and PP2A activity in lateral habenula contributes to amplification of its firing bursts activity and subsequent inhibition of monoaminergic reward centers, therefore contributing to depressive symptomatology. This research suggests that parallel cellular processes converge in the lateral habenula to transduce the impact of aversive stimuli.

KEY CONCEPTS

1. Aversive and invalidating environmental stimuli individually or coupled with chronic stress precipitate increased firing of the lateral habenula, leading to down-regulation of brainstem dopaminergic neurotransmission and decreased activity of the nucleus accumbens, inducing anhedonia and depressive symptomatology.
2. Increased lateral habenular firing also leads to down-regulation of the serotonergic neurons in raphe nuclei neurons and decreased serotonergic neurotransmission, known to be implicated in depressive symptomatology.
3. In contrast, outcomes that meet or exceed expectations lead to decreased lateral habenular firing and increases in dopaminergic and serotonergic neurotransmission. The ability to update expectations of the environment based on rewards and disappointments, reflects a potentially important survival mechanism relevant to the capacity to adapt to ever-changing environmental circumstances.
4. Multiple lines of evidence derived by both preclinical and clinical studies confirm the relationship between stimuli that activate the firing rate of the lateral habenula and the appearance of depressive symptomatology. Postmortem studies clinical finding of a smaller lateral habenula in depressed patients, while human clinical imaging studies show increased glucose utilization of this structure, which normalizes following ketamine treatment.
5. Broadly, ketamine-induced rapid antidepressant and antianhedonic effects are believed to be mediated by a) direct inhibition of spontaneous synaptic NMDAR in the prefrontal cortex and hippocampus or inhibition of NMDAR-burst activity in lateral habenula, b) indirect inhibition of NMDAR at a presynaptic GABAergic interneuron site, or c) conversion of ketamine to its active metabolite HNK, inducing an early and sustained activation of AMPAR at hippocampus, proving for the first time that NMDAR inhibition is not essential for the antidepressant effects of ketamine.
6. Direct injection of ketamine into the lateral habenula, an anti-reward center abundant in NMDA receptors, induces rapid amelioration of experimentally induced depressive-like syndromes in animal models.
7. Aversive stimuli induce up-regulation of the Kir4.1 or p11 is associated with rapid firing rates in the lateral habenula and induces depression-like phenotype, while pharmacological down-regulation or knockout manipulation in animal models is associated with resolution of depression-like symptoms.
8. Increasing the presence of the enzyme βCaMKII consistently increased depressive behaviors, including anhedonia and behavioral despair. In contrast, antidepressant medications and RNA interference of βCaMKII significantly diminished depressive manifestations.
9. Chronic stress causes significant weakening of $GABA_B$–GIRK pathway function and neuronal excitability in lateral habenula, which is restored by pharmacologic inhibition of PP2A. Thus, PP2A inhibitors may have therapeutic efficacy in depressive syndromes associated with increased firing of lateral habenula neurons.
10. The HPA axis and the lateral habenula appear to be intricately interconnected to regulate the stress-related adaptive response to aversive stimuli.
11. Increase in lateral habenula firing rate induces decrease in slow wave activity through the influence of serotonin neurons in the VTA and raphe nuclei, whereas the augmentation of REM sleep activity appears to be mediated by descending brainstem circuits *via* RMTg.
12. Strategies aimed at decreasing the firing rate of the lateral habenula show great promise for significant amelioration of associated depressive symptomatology.

AUTHOR CONTRIBUTIONS

PG and BK designed the contents of this article, interpreted the data, and wrote the review. Both authors read and approved the final manuscript.

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SYSTEMATIC REVIEW
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Glucocorticoid Resistance: Is It a Requisite for Increased Cytokine Production in Depression? A Systematic Review and Meta-Analysis

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Background: Glucocorticoid resistance—reduced function of the glucocorticoid receptor (GR)—is seen in many depressed patients. It is argued that this resistance to glucocorticoids leads to failure of normal feedback regulation on the immune system. High levels of pro-inflammatory cytokines result.

Purpose: We sought to identify evidence supporting or refuting a link between glucocorticoid resistance and immune dysregulation in depression and to summarize retrieved evidence in aggregate form.

Methods: We systematically reviewed and meta-analyzed studies that examined cytokine levels in depressed patients compared with controls and that also reported a measure of glucocorticoid resistance. These measures included plasma cortisol, the dexamethasone suppression test (DST), *GR* expression levels, and the results of *in vitro* assays of GR function. We conducted four separate meta-analyses to test for moderating effects of glucocorticoid resistance on cytokine production in depression.

Results: After sub-grouping 32 studies by the ratio of cortisol levels in patients compared with controls, we observed a trend for increasing glucocorticoid resistance (i.e., the most hypercortisolemic patients) to be associated with increased production of interleukin (IL)-6 [d = 0.94; 95% CI (0.29, 1.59)] and tumour necrosis factor (TNF)-α [d = 0.46; 95% CI (0.12, 0.79)]. We stratified nine studies that reported DST results by relative glucocorticoid resistance between patients and controls, identifying a trend for higher glucocorticoid resistance in patients, compared with controls, to be associated with higher cytokine production in patients (170 patients and 187 controls). This was particularly evident when studies were sub-grouped by source of cytokine—plasma (d = 1.04; 95% CI, 0.57–1.50) versus *in vitro* (d = 0.24; 95% CI, –0.20 to 0.67). Stratifying the four studies (147 patients and 118 controls) that used *in vitro* assays of GR function or *GR* expression to quantify glucocorticoid resistance revealed variable contributions to cytokine production in patients compared with controls (overall effect size: d = 1.35; 95% CI 0.53–2.18). Combining our

analyses of studies that reported DST results with those that used *in vitro* assays of GR function or *GR* expression to quantify glucocorticoid resistance (302 patients and 277 controls), we noted that although depressed patients produced more cytokines than controls (d = 1.02; 95% CI, 0.55–1.49), there was no evident positive correlation between glucocorticoid resistance and inflammation.

Conclusions: Our work provides some support for a model conceptualizing glucocorticoid resistance as a requisite for increased inflammation in depression. The limited number of studies identified highlights the need for purpose-designed investigations that directly examine the relationship between glucocorticoid resistance and cytokine production in depression.

Keywords: depression, cytokines, glucococorticoids, glucocorticoid resistance, inflammation

INTRODUCTION

Endogenous glucocorticoids play an essential role in driving adaptive responses to stress. They increase available blood glucose and initiate lipolysis for increased metabolic demands under stress, alter behavioral responses to stress, and modulate stress-induced immune function to prevent overactivation and consequent damage to host tissues (1, 2).

Secretion of endogenous glucocorticoids is tightly controlled by the hypothalamic–pituitary–adrenal axis (HPA axis). Corticotropin releasing factor (CRF) produced in the periventricular nucleus of the hypothalamus triggers release of adrenocorticotropin (ACTH) by the anterior pituitary. ACTH in turn triggers release of glucocorticoids, especially cortisol in humans, from the adrenal cortex (3). Importantly, secreted glucocorticoids engage feedback mechanisms in the anterior pituitary and the hypothalamus to limit further secretion of ACTH and CRF, respectively.

A large body of work has identified that in diseases of chronic stress, disruptions in the normal regulation of the HPA axis are present. In depressed patients, increased cortisol levels (4) that are resistant to feedback regulation by the HPA axis have been detected (5, 6). Similar increases in glucocorticoids and disruptions of HPA axis regulation in non-depressed patients are associated with the Cushing syndrome of glucocorticoid excess (7), yet depressed patients with elevated cortisol levels do not manifest the same syndrome. This observation argues for the presence of a resistance to high glucocorticoid levels in depressed patients. Multiple mechanisms have been invoked to explain this, including: impairments in glucocorticoid receptor (GR) function, changes in *GR* expression, alterations in glucocorticoid bioavailability through modified protein binding in the serum, changes in the HPA axis feedback rheostat, and impacts on the ability of the immune system to modulate glucocorticoid function (3). Indeed, proinflammatory cytokines can also feedback on the hypothalamus and anterior pituitary, for example, at times increasing HPA axis activity through modulation of GR function and expression (3, 8).

A reciprocal relationship between glucocorticoids and immune function also exists—high levels of glucocorticoids are known to strongly inhibit immune function. This well-known property of glucocorticoids is exploited in the clinical treatment of inflammatory and autoimmune diseases (2, 9) and is believed to play a role in protecting the nervous system from an over-active inflammatory response (10). In depression, high levels of glucocorticoids can co-exist with high levels of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α (8, 11). The concurrent presence of high glucocorticoid levels and high cytokine levels in depressed patients creates a complex interplay between the immune system and the HPA axis. For example, high levels of glucocorticoids would be expected to dampen immune function and cytokine elaboration, but they do not always. Models to explain this phenomenon suggest that high levels of glucocorticoids in depression cause resistance to glucocorticoid feedback on the HPA axis and that this developed glucocorticoid resistance allows the escape of pro-inflammatory signaling pathways from normal feedback inhibition (11) through the mechanisms discussed above.

Many studies have focused on characterizing either elevated glucocorticoids or cytokine-mediated inflammation in depression. Few works have focused on the relationship between glucocorticoid resistance and inflammation in depression, and the few that have produced conflicting results. We were interested in exploring this relationship further and hoping to resolve inconsistencies between the results of individual studies. Therefore, we conducted a systematic review and meta-analysis of all published studies that simultaneously reported the results of indices of glucocorticoid resistance and cytokine levels in depression. Such indices of glucocorticoid resistance included plasma cortisol (4), the dexamethasone suppression test (DST) (12), *GR* expression (11), and *in vitro* functional assays of the GR (13). Classically in depression, plasma cortisol is elevated, and dexamethasone is unable to restore this elevation to normality. *GR* expression is downregulated, and *in vitro* functional assays of the GR show resistance to exogenous glucocorticoid actions. Using this knowledge, we examined for links between glucocorticoid resistance and the elaboration of high levels of pro-inflammatory cytokines in depression and summarized these results to produce aggregate effects. We hoped to identify enough aggregate evidence to allow us to resolve the conflict present in the primary literature.

METHODS

We conducted our search and review using the methods outlined by Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (14).

Search Strategy

We reasoned that evidence of glucocorticoid resistance may manifest by either of three outcomes in depressed patients—elevated plasma cortisol compared with control; greater proportional abnormalities on the DST/other endocrine suppression test(s) compared with control; or evidence of glucocorticoid resistance only *in vitro* or through reduced *GR* expression compared with control.

Endocrine suppression tests assess the ability of exogenously supplied glucocorticoids to suppress endogenous effects of cortisol and other glucocorticoids. The DST is the most commonly used of such assays. The DST identifies glucocorticoid resistance in subjects by examining the ability of exogenously supplied dexamethasone to suppress plasma cortisol levels *in vivo*. Subjects in whom serum cortisol levels do not reduce following dexamethasone administration are classified as "non-suppressors" and display *in vivo* evidence of glucocorticoid resistance (12).

Evidence of glucocorticoid resistance in blood or *ex vivo* cells is determined using a similar premise, namely, the ability of exogenous glucocorticoids to suppress *in vitro* proliferation or function of immune cells isolated from depressed patients and controls, or by reduced *GR* expression in depressed patients compared with controls.

To ensure that all three manifestations of glucocorticoid resistance were explored, we conducted three separate yet complementary literature searches to assess all eventualities.

i) Glucocorticoid Resistance as Assessed by Elevated Plasma Cortisol in Depressed Patients, Compared to Controls

MEDLINE, EMBASE, PsycInfo, and the Cochrane Database were searched for articles from origin until October 31, 2018, using the following search strategy: (exp Depression OR exp Depressive Disorder) AND (exp Adrenal Cortex Hormones OR exp Pituitary-Adrenal System) AND (exp Cytokines OR exp Inflammation Mediators OR exp Leukocytes OR exp Macrophages). Results were limited to studies in humans and reported in English. Inclusion criteria were as follows: studies that reported a measure of cortisol (preferably morning) in each adult (age > 17 years) depressed patient and control, as well as corresponding cytokine levels (measured either from blood or from *in vitro* studies of blood or immune cells) in both subject populations, and depression diagnosed in patients using a standardized clinical/diagnostic interview. Criteria leading to study exclusion included the following: presence of bipolar, psychotic or substance use disorders in patients or controls; acute infection in patients or controls within 2 weeks of the study; and obesity [body mass index (BMI) > 30] in patients or controls.

Anticipating that a low number of studies would be retrieved by our search, we did not exclude studies in which patients or controls suffered from chronic medical conditions (e.g., endocrine, inflammatory, autoimmune, oral-dental, or neurologic disease) or in which immune-modulating therapies, such as glucocorticoids or biologics, were used in patients or controls so long as only a minority of subjects (<10%) possessed either of these respective characteristics, or in which matched controls, including matching for chronic medical conditions and immune modulating therapies, were used. We also did not exclude studies in which depressed patients experienced co-morbid anxiety disorders, so long as the anxiety disorder was not the primary diagnosis at the time of study.

The search was executed by AP. Retrieved titles and abstracts were screened by AP and MH to assess conformity with inclusion and exclusion criteria. Disputes about the appropriateness for study inclusion were resolved by AP. Articles deemed suitable for inclusion were retrieved in full text and examined by AP. Reference lists of retrieved articles were also examined to identify additional relevant articles not identified in our database searches.

ii) Glucocorticoid Resistance Measured by DST or Other Endocrine Suppression Test in Patients and Controls

A similar search strategy was employed to that above. Identical databases for the same periods were queried using the following search terms: (exp Depression OR exp Depressive Disorder OR exp Affective Symptoms) AND (exp Hydrocortisone OR exp Glucocorticoids OR exp Adrenal Cortex Hormones OR exp Pituitary-Adrenal System) AND (exp Receptors, Steroid OR exp Dexamethasone OR suppression test.mp. OR glucocorticoid resistance.mp). AND (exp Inflammation OR exp Inflammation Mediators OR exp Cytokines OR exp Leukocytes OR exp Macrophages). Results were again limited to studies in humans and reported in English. Inclusion criteria were as follows: studies that reported a result from an endocrine suppression test in each adult (age > 17 years) depressed patient and control, as well as corresponding cytokine levels (measured either from blood or from *in vitro* studies of blood or immune cells) in both subject populations; and depression diagnosed in patients using a standardized clinical/diagnostic interview. Criteria leading to study exclusion were the same as those enumerated above. The search was executed, and articles screened and retrieved as described above.

iii) Glucocorticoid Resistance Measured Only *In Vitro* or by GR Expression

Identical databases for the same periods as above were queried using the following search terms: (exp Depression OR exp Depressive Disorder) AND (exp Adrenal Cortex Hormones OR exp Pituitary-adrenal System OR Steroid Receptor) AND (exp Cytokines OR exp Inflammatory Mediators OR exp Leukocytes OR exp Macrophages). Search results were limited as above. Inclusion criteria were: studies that reported a result from an *in vitro* assay of GR function, such as suppression of *in vitro* proliferation of immune cells by exogenous glucocorticoids or measurement of cytokine production as an assay for GR function in blood, or *GR* expression in each adult (age > 17) depressed patient and control, as well as corresponding cytokine levels (measured from plasma) in both subject populations; and depression diagnosed in patients using a standardized clinical/diagnostic interview. Criteria leading

to study exclusion were the same as above. The search was executed, and articles screened and retrieved as described above.

Data Extraction

Means and standard deviations for individual cytokine level from depressed patients and controls were extracted from studies when reported. When such values were not reported, we contacted study authors to obtain either raw data or the necessary values. When there were gaps in the data set of a given study (i.e., not all subjects had reported a measure of glucocorticoid resistance and a measure of cytokine level) and we were unable to obtain additional data from the study authors to bridge these gaps, we included these studies as they represented a minority in our analysis. Such inclusions are noted in the presented summary tables.

In some studies, values for plasma cortisol and cytokines were not normally distributed. We estimated mean and standard deviations from reported medians, data ranges, and sample sizes using the method of Wan (15). Non-Gaussian data has been found to have limited impact on the outcomes of meta-analysis (16), and given the small number of studies retrieved, we felt that excluding such data would materially bias our analysis.

When depressed subjects were divided into subtypes of the illness (i.e., with atypical features, with melancholic features, etc.), we combined all listed sub-types into one group of depressed patients and calculated means and standard deviations for these single groups. Where studies reported data on more than one patient group (e.g., patients with depression and another disease, as well as patients with depression only), we extracted data for patients with depression only, unless matched controls were used.

In the case of endocrine suppression test results, we extracted counts of suppressors and non-suppressors from reported studies or unpublished data provided by study authors. Although the plasma cortisol level used to define non-suppression in the DST varied from study to study, all cut-offs exceeded the generally accepted value of 1.8 μg/dL (17). If a study used a significantly higher cut-off value, we did not modify suppression and non-suppression counts as for such studies we did not possess the raw post-dexamethasone cortisol values that would have allowed us to make such modifications.

In vitro studies commonly reported outcomes for assays of GR function as percentage of basal effect. We extracted the difference of these percentages from 100% for further use in our study. *GR* expression levels from whole blood were reported as fold-change and extracted as such.

Meta-Analysis

Meta-analyses were conducted using RevMan5 (18) and effect sizes are reported as Cohen's *d*. Cohen's *d* is calculated as follows:

$$\frac{\bar{x}_{\text{depressed}} - \bar{x}_{\text{control}}}{\sqrt{\frac{\sigma^2_{\text{depressed}} - \sigma^2_{\text{control}}}{2}}}$$

We derived effect sizes from means and standard deviations of cytokine levels from depressed patients and controls. Where reported, we preferentially used plasma values of cytokines in our analysis. When stimulated cytokine levels from *in vitro* assays were used, we selected the stimulant level at which maximal response was noted by the study authors.

Since we identified experimental variability during our systematic review, we presumed that there would be heterogeneity in our meta-analysis attributable to this variability and therefore conducted analysis using a random effects model.

A priori, we hypothesized that relative glucocorticoid resistance differences between patients and controls would also contribute to the heterogeneity observed between studies. Thus, we constructed two measures of relative glucocorticoid resistance that would allow us to undertake modifier analysis.

i) Glucocorticoid Resistance as Assessed by Relative Plasma Cortisol Levels Between Depressed Patients and Controls

We presumed that glucocorticoid resistance would manifest by high levels of plasma cortisol. We therefore used the ratio of average plasma cortisol in patients to average plasma cortisol in controls to assess relative glucocorticoid resistance between the two subject groups. Subsequent modifier analysis sub-grouped effect sizes from studies into those studies where patients were hypercortisolemic compared to controls (ratio patient:control > 1.2), patients had essentially similar plasma cortisol levels to controls (ratio, 0.8 < patient:control < 1.2; "eucortisolemia"), and where patients displayed lower plasma cortisol levels than controls (ratio patient:control < 0.8; "hypocortisolemia"). This modifier analysis allowed us to examine effect sizes in studies in which patients may have displayed more glucocorticoid resistance than in controls and to compare these with effect sizes from studies where there was a reduced relative difference in presumed glucocorticoid resistance between patients and controls.

Since we decided to use such an approach in our meta-analysis, we report pooled effect sizes only for those cytokines whose values were reported by five or more retrieved studies.

ii) Glucocorticoid Resistance Measured by DST or Other Endocrine Suppression Test in Patients and Controls, or in *In Vitro* Studies of GR Function in Blood or Cells from Patients and Controls or from GR Expression Levels in the Blood of Patients and Controls

To assess relative differences in glucocorticoid resistance between patients and controls when an endocrine suppression test was used, we developed a continuous measure of this comparison—the "glucocorticoid resistance index."

$$\frac{\text{proportion of suppressors}_{\text{control}} - \text{proportion of suppressors}_{\text{patients}}}{\text{proportion of suppressors}_{\text{control}}}$$

This measure of relative difference in glucocorticoid resistance varies between −1 (all controls glucocorticoid resistant and none of patients) and 1 (all patients glucocorticoid resistant and none of controls). Such an approach avoids the mathematical difficulties inherent to

comparing numbers of non-suppressors in patients and controls. We used the "glucocorticoid resistance index" as a variable to rank retrieved studies by the relative difference in glucocorticoid resistance between patients and controls (i.e., studies listed first in **Figures 4**, **5**, and **6** are those in which most, if not all, patients are glucocorticoid resistant and few, if any, controls are glucocorticoid resistant).

To analyze relative glucocorticoid resistance using *in vitro* measures of GR function, we modified the "glucocorticoid resistance index" as follows:

$$\frac{[1-\text{proportion of basal}_{\text{control}}]-[1-\text{proportion of basal}_{\text{patients}}]}{[1-\text{proportion of basal}_{\text{control}}]}$$

where "basal" is assay output in the absence of exogenous glucocorticoid. This measure varies in an identical manner to the classic "glucocorticoid resistance index." When reported, we preferentially used these measures of GR function instead of *GR* expression.

To analyze relative glucocorticoid resistance using *GR* expression, we modified the "glucocorticoid resistance index" as follows:

$$\frac{\text{GR expression}_{\text{control}}-\text{GR expression}_{\text{patients}}}{\text{GR expression}_{\text{control}}}$$

This measure varies identically to those discussed above.

As we retrieved insufficient studies to conduct meta-analysis by individual cytokine in this arm of analysis, we selected from each study the cytokine reported (if more than one was reported) using the following prioritization criteria: 1) for studies reporting an endocrine suppression test result in patients and controls, cytokines for which most of the other studies also reported a value; barring this, cytokines for which a plasma level, rather than an *in vitro* level, was reported; 2) for studies reporting an *in vitro* measure of GR function or *GR* expression in patients and controls, cytokines for which most of other studies also reported a value from plasma; barring this, cytokines for which the maximum positive effect size was demonstrated in plasma.

Assessment of Heterogeneity

Heterogeneity in pooling effects sizes was first assessed visually on forest plots. Standard assessments of heterogeneity calculated by RevMan (τ^2 and I^2) were further used to assess the contribution of heterogeneity between studies to overall appropriateness in pooling effect sizes. τ^2 and I^2 were used to examine the impact of moderator analysis on pooled effects sizes generated in sub-group analysis.

Sensitivity Analysis and Reporting Bias

We conducted standard serial exclusion of studies to assess for individual study effect on the overall effect size reported. Funnel plots were generated in RevMan (18).

RESULTS

As three separate yet complementary approaches were utilized to examine our question, we report results for each approach serially.

i) Glucocorticoid Resistance as Assessed by Relative Plasma Cortisol Levels Between Depressed Patients and Controls

There were 3,328 articles identified in our database search (**Figure 1**). After removal of duplicates and review of titles and abstracts to ensure that studies met our inclusion criteria, 45 articles were retrieved for full-text review. Twelve articles were excluded for the following reasons: 2 studies did not include a control group; 5 studies did not report a measure of cortisol; 1 study reported cytokine levels only after oral dexamethasone challenge of patients and controls; 1 study did not report cytokine measures; 1 study did not use a structured clinical/diagnostic interview to diagnose depression in patients or to exclude mental illness in controls; 2 studies included patients who suffered from bipolar, psychotic, or substance use disorders.

Further detailed review of the remaining 33 articles identified two articles that examined the same cytokine in the same patient population. We excluded one of these articles (19) to avoid duplication bias and included the other, which reported a more comprehensive analysis of the subjects (20). This left 32 articles to include in our review (**Table 1**).

All studies were either of a case–control design or a non-randomized cohort design. For the latter type of study, we extracted data only from the baseline timepoint. This removed the impact of treatment interventions and effectively transformed the extracted data into a case–control design.

All studies reported either plasma or salivary cortisol levels in patients and controls. One study reported both plasma and salivary cortisol as well as *GR* expression (45). Thirty (94%) studies collected blood or saliva for cortisol analysis in the morning, generally between the hours of 0700 and 1100 (**Table 1**). Of the two remaining studies, one collected samples for cortisol analysis in the afternoon (50) and the other did so in the evening (47). Nine studies specifically mentioned a rest period of 15 to 45 min prior to the collection of samples used for quantification of cortisol (20–22, 23, 29, 35, 41, 44, 50). The remaining studies did not comment on this subject. Only two studies reported cortisol awakening responses in the form of area under the curve with respect to ground (AUC_g) as their cortisol outcome measure (37, 49). All other studies reported average cross-sectional cortisol levels at the time specified in **Table 1**.

Twenty-four (75%) studies reported plasma, blood or salivary levels of cytokines measured by either ELISA or mRNA expression. The remaining studies reported only cytokine or mRNA levels from *in vitro* analysis of whole blood or immune cells. For the 24 studies that reported plasma, blood or salivary levels of cytokines, 23 either collected cytokine samples concomitantly with cortisol samples or within 1 h of the collection of cortisol samples. The remaining study (47) collected cytokine samples at 1200 and cortisol samples between 1900 and 2200.

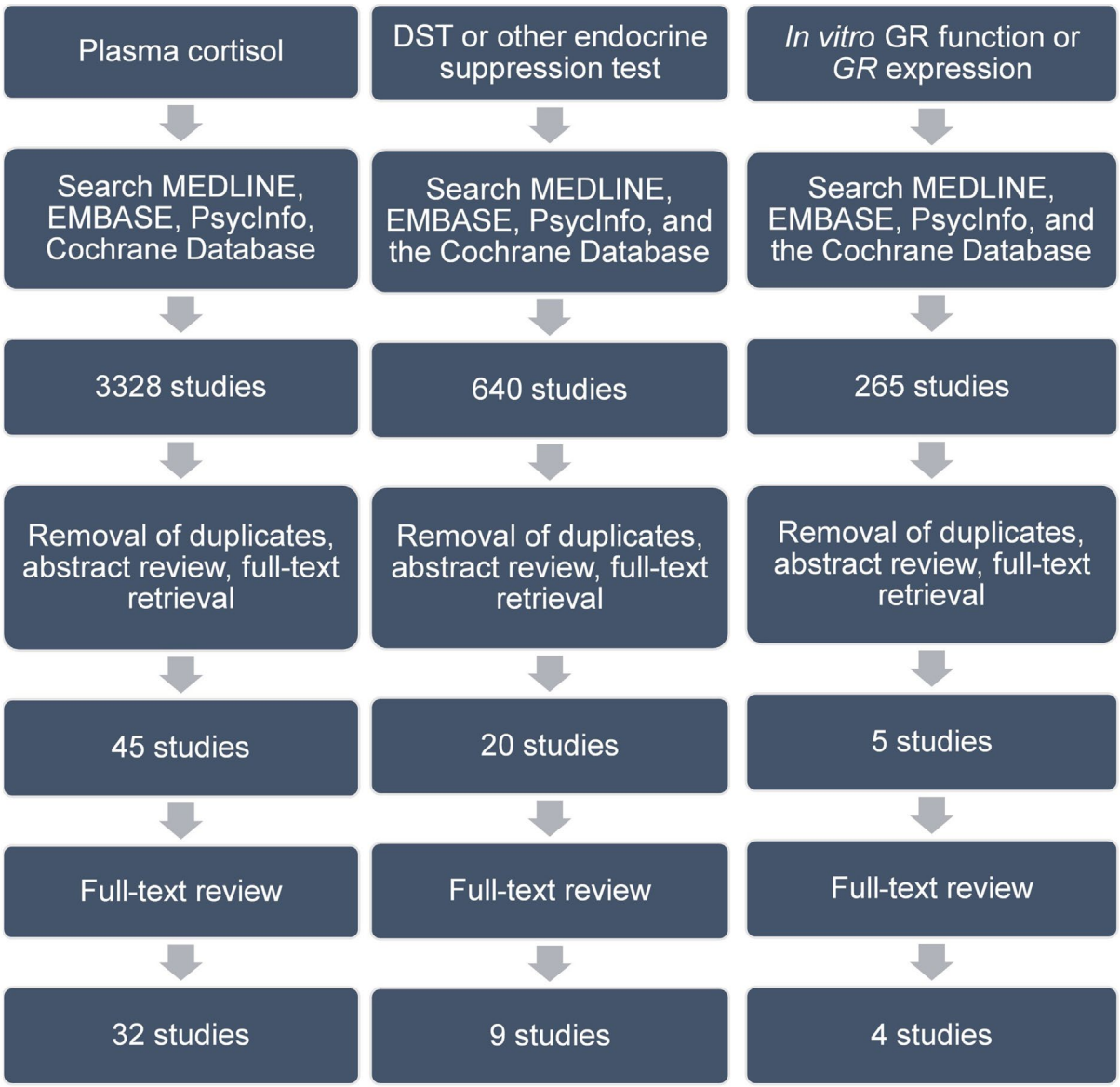

FIGURE 1 | Search strategy and article review process. Details of this are found in text. Abbreviations are as follows: DST, dexamethasone suppression test; GR, glucocorticoid receptor.

Of note, 29 (91%) studies reported results from patients and controls with no psychiatric nor medical co-morbidities. Two studies were conducted exclusively in the elderly (age range, 50–90 years) who displayed a number of medical co-morbidities, such as coronary artery disease, diabetes, osteoarthritis, and major vascular neurocognitive disorder (45, 48). Patient and control groups in these studies were equally matched for medical and psychiatric co-morbidities, including neurocognitive disorders (48).

Antidepressant medications were used in patients in 69% of reviewed studies. Only three studies included patients who were using anti-inflammatory medications including glucocorticoids (doses not reported), but in two of these studies, less than 5% of patients used these medications (37, 49), and in the other study (48), anti-inflammatory medication use was negated by the use of matched controls. A small number of studies (13%) had gaps in the reported data that could not be rectified by attempted contact with study authors (21, 43, 48, 49).

Seven studies reported C-reactive protein (CRP) levels (20, 37, 44, 45, 47, 49, 50), one study reported eosinophil cationic protein (ECP) levels (27), one study reported eosinophil chemotactic protein-2 (EOTAXIN-2) levels (27), five studies reported interferon-γ (IFN-γ) levels (22, 27, 36, 39, 43), six studies reported IL-1β levels (23, 31, 38, 40, 43, 46), two studies reported IL-1 receptor antagonist (IL-1RA) levels (31, 47), five studies reported IL-2 levels (23, 24, 26, 30, 39), two studies reported

TABLE 1 | Studies included in analysis using relative plasma cortisol levels as a measure of glucocorticoid resistance.

Study	Patients	Control	Age	Medical co-morbidity	Psychiatric co-morbidity	Medications in patients	Anti-inflammatories	Diagnostic method	Cortisol source; sample timing/ detail	Cortisol level patients	Cortisol level control	Cortisol ratio—patients/ control	Cytokine; source; sample timing/ detail	Stimulant	Cytokine level patient	Cytokine Level Control
Alesci (21)[a]	9 outpatients	9, matched	Adult	No	No	Yes	No	SCID-IV	Plasma; at 0800/after 30-min rest	9.7 +/– 1.0 (SEM) μg/dL	11.4 +/– 0.8 (SEM) μg/dL	0.85	IL-6; plasma; 0900/after 60-min rest	None	5.3 +/– 1.5 (SEM) pg/ml	3.4 +/– 0.6 (SEM) pg/ml
Allen (22)	37 combined inpatients and outpatients	20	Adult	No	No	Yes	No	SCID-IV	Salivary; 30-min post-awakening/ variable waking time	12.24 +/– 3.55 (SD) nM	10.5 +/– 1.5 (SD) nM	1.16	IL-6, IL-8, IL-10, IFN-γ; plasma; between 0800 and 1100	None	IL-6—2.72 +/– 1.67 (SD) pg/ml; IL-8— 2.23 +/– 3.55 (SD); IL-10 – 7.27 +/– 2.73 (SD) pg/ml; IFN-γ—17.7 +/– 4.27 (SD) pg/ml	IL-6—2.8 +/– 0.3 (SEM) pg/ml; IL-8 - 11 +/– 1.0 (SEM) pg/ml; IL-10 - 6.5 +/– 1.0 (SEM) pg/ml; IFN-γ—15.2 +/– 2.3 (SEM) pg/ml
Anisman (23)	45 outpatients	27	Adult	No	No	No	No	Patient—clinical interview; control—MINI	Plasma; between 0730 and 0900/after 20-min rest	12.61 +/– 3.59 (SD) μg/dL	16.18 +/– 5.45 (SD) μg/dL	0.78	IL-1β, IL-2; *ex vivo* cells stimulated	PHA	IL-1β—1,281.1 +/– 36.19 (SD) μg/ml; IL-2—512.89 +/– 22.9 (SD) pg/ml	IL-1β—1,400 +/– 519.62 (SD) μg/ml; IL-2—980 +/– 519.62 (SD) pg/ml
Bauer (24)	36 inpatients	31	Adult	No	No	Yes	No	Patient—clinical interview; control—clinical interview	Salivary; at 1000/ prior to phlebotomy	11.88 +/– 3.5 (SD) nM	9.1 +/– 5.7 (SD) nM	1.31	IL-2, TNF-α; *ex vivo* cells stimulated	PHA (IL-2), LPS (TNF-α)	IL-2—338.5 +/– 69.8 (SEM) pg/ml; TNF-α—880 +/– 90 (SEM) pg/ml	IL-2 297.1 +/– 101.7 (SEM) pg/ml; TNF-α—890 +/– 90 (SEM) pg/ml
Carvalho (25)	19 inpatients	21	Adult	No	No	Yes	No	SCID-IV	Plasma; morning	340 +/– 30 (SEM) pg/ml	200 +/– 20 (SEM) pg/ml	1.7	IL-4, IL-6, IL-10, MCP-1, TNF-α, VEGF; plasma; morning	None	IL-4—2.6 +/– 0.1 (SEM) pg/ml; IL-6 - 3.0 +/– 0.1 (SEM) pg/ml; IL-10—1.7 +/– 0.05 (SEM) pg/ml; MCP-1—150 +/– 15 (SEM) pg/ml; TNF-α—2.9 +/– 0.1 (SEM) pg/ml; VEGF—14.5 +/– 1.5 (SEM) pg/ml	IL-4—3.2 +/– 0.5 (SEM) pg/ml; IL-6 - 1.9 +/– 0.15 (SEM) pg/ml; IL-10—1.3 +/– 0.05 (SEM) pg/ml; MCP-1 - 120 +/– 10 (SEM) pg/ml; TNF-α- 2.3 +/– 0.2 (SEM) pg/ml; VEGF - 23 +/– 2 (SEM) pg/ml
Carvalho (13)	15, inpatients	28	Adult	No	No	Yes	No	Patient—SCID–IV; control – not specified	Plasma; at 1000	429.4 +/– 55.4 (SEM) nM	242.2 +/– 14.8 (SEM) nM	1.77	IL-6; plasma and whole blood stimulated; at 1000 (plasma)	LPS (whole blood)	Plasma—3.0 +/– 0.29 (SEM) pg/ml; whole blood stimulated—1,025 +/– 175 (SEM) pg/ml	Plasma—2.4 +/– 0.1 (SEM) pg/ml; whole blood stimulated—875 +/– 150 (SEM) pg/ml
Cubala (20)[b]	20 outpatients	20	Adult	No	No	No	No	SCID-IV	Plasma; between 0800 and 0900/after 45-min rest	426.95 (369.2, 484.6) (95% CI) nM	322 (264.5, 379.5) (95% CI) nM	1.33	CRP; salivary; between 0800 and 0900/after 45-min rest	None	108.07 +/– 97.74 (SD) pg/ml	115.7 +/– 80.18 (SD) pg/ml
Darko (26)	20 inpatients	20	Adult	No	No	No	No	SCID-III	Plasma; between 0830 and 0930	20 +/– 5 (SD) μg/dL	16 +/– 5 (SD) μg/dL	1.25	IL-2; *ex vivo* cells stimulated	PHA	3.3 +/– 6.0 (SD) IU/ml	3.0 +/– 3.2 (SD) IU/ml

TABLE 1 | Continued

Study	Patients	Control	Age	Medical co-morbidity	Psychiatric co-morbidity	Medications in patients	Anti-inflammatories	Diagnostic method	Cortisol source; sample timing/ detail	Cortisol level patients	Cortisol level control	Cortisol ratio—patients/ control	Cytokine; source; sample timing/ detail	Stimulant	Cytokine level patient	Cytokine Level Control
Du (27)	21 outpatients	27	Adult	No	No	Not specified	No	Clinical interview	Salivary; at 0800	8.6 +/– 2.4 (SEM) pg/μL	8.4 +/– 1.5 (SEM) pg/μL	1.02	ECP, EOTAXIN-2, IFN-γ, RANTES, TNF-α; plasma; at 0800	None	ECP—8.9 +/– 0.6 (SEM) μg/L; EOTAXIN-2 – 306.9 +/– 72.7 (SEM) pg/ml; IFN-γ—149.5 +/– 10.1 (SEM) pg/ml; RANTES—3368.0 +/– 129.6 (SEM) pg/ml; TNF-α—132.3 +/– 9.8 (SEM) pg/ml	ECP—12.5 +/– 1.9 (SEM) μg/L; EOTAXIN-2—383.6 +/– 84.0 (SEM) pg/ml; IFN-γ—143.8 +/– 6.7 (SEM) pg/ml; RANTES—3,410.8 +/– 113.9 (SEM) pg/ml; TNF-α—126.8 +/– 8.8 (SEM) pg/ml
Fitzgerald (28)	19	38	Adult	No	No	Yes	No	Patient—clinical interview; control—not specified	Plasma; between 0900 and 1100	325.5 +/– 26.4 (SEM) nM	294.6 +/– 28.3 (SEM) nM	1.1	IL-6; TNF-α; plasma; between 0900 and 1100	None	TNF-alpha—22.02 +/– 3.62 picogram/ml (mean +/– SEM); *n* = 19, IL-6—1.18 +/– 0.12 picogram/ml; n = 19	TNF-alpha—12.10 +/– 2.56 picogram/mL (mean +/– SEM); n = 38, IL-6—0.73 +/– 0.11 picogram/ml; *n* = 38
Humphreys (29)	9 outpatients	11	Adult	No	No	No	No	Patient—SCID-IV; control—not specified	Plasma; at 0800/after 30-min rest	20.1 +/– 3.7 (SEM) μg/dL	19.5 +/– 7.7 (SEM) μg/dL	1.03	IL-6; *ex vivo* cells unstimulated and stimulated	LPS	Unstimulated—3,541.2 +/– 726.8 (SEM) pg/ml; stimulated – 19,867.7 +/– 3,649.2 (SEM) pg/ml	Unstimulated—380.4 +/– 77.5 (SEM) pg/ml; stimulated—33,142.2 +/– 1,547.2 (SEM) pg/ml
Jozuka (30)	17 outpatients	10	Adult	No	No	No	No	Clinical interview	Plasma; between 0900 and 1000	7.1 +/– 4.5 (SD) μg/dL	12.3 +/– 3.8 (SD) μg/dL	0.58	IL-2; plasma; between 0900 and 1000	None	542 +/– 111 (SD) pg/ml	344 +/– 98 (SD) pg/ml
Kaestner (31)	37 inpatients	37	Adult	No	No	No	No	SCID-IV	Plasma; at 0800	203.51 +/– 14.46 (SD) ng/ml	180 +/– 80 (SD) ng/ml	1.13	IL-1β, IL-1RA; plasma; at 0800	None	IL-1β—37.3 +/– 6.19 (SD) pg/ml; IL-1RA—2,224.32 +/– 47.81 (SD) pg/ml	IL-1β—21 +/– 27 (SD) pg/ml; IL-1RA—1,600 +/– 750 (SD) pg/ml
Kahl (32)	34 outpatients	25	Adult	No	No	Yes	No	Patient—SCID-IV; controls—standardized psychiatric interview	Plasma; between 0700 and 0800	579.1 +/– 162.6 (SD)	423.4 +/– 150.1 (SD) nM	1.37	IL-6, TNF-α; plasma; between 0700 and 0800	None	IL-6—1.5 +/– 0.8 (SD) pg/ml; TNF-α—1.7 +/– 1.5 (SD) pg/ml	IL-6—1.7 +/– 1.4 (SD) pg/ml; TNF-α —0.7 +/– 0.5 (SD) pg/ml
Kahl (33)	27 inpatients	19	Adult	No	No	Yes	No	Patient—SCID-IV; control—standard psychiatric interview	Plasma; between 0700 and 0800	556.7+/– 150.5 (SD) nM	412.3 +/– 123.4 (SD) nM	1.35	IL-6, TNF-α; plasma; between 0700 and 0800	None	IL-6—1.9 +/– 2.2 (SD) pg/ml; TNF-α—1.9 +/– 1.8 (SD) pg/ml	IL-6—1.8 +/– 1.4 (SD) pg/ml; TNF-α —0.8 +/– 0.5 (SD) pg/ml

TABLE 1 | Continued

Study	Patients	Control	Age	Medical co-morbidity	Psychiatric co-morbidity	Medications in patients	Anti-inflammatories	Diagnostic method	Cortisol source; sample timing/ detail	Cortisol level patients	Cortisol level control	Cortisol ratio—patients/ control	Cytokine; source; sample timing/ detail	Stimulant	Cytokine level patient	Cytokine Level Control
Kahl (34)	18	20	Adult	No	No	Yes	No	Patient—SCID-IV; control—standardized psychiatric interview	Plasma; between 0700 and 0800	661 +/– 384 (SD) nM	554 +/– 119 (SD) nM	1.19	IL-6, TNF-α; plasma; between 0700 and 0800	None	IL-6—1.45 +/– 1.8 (SD) pg/ml; TNF-α—3.90 +/– 0.9 (SD) pg/mL	IL-6—0.76 +/– 0.33 (SD) pg/ml; TNF-α—1.99 +/– 0.51 (SD) pg/mL
Karlovic (35)	55 inpatients	18	Adult	No	No	Yes	No	SCID-IV	Plasma; between 0800 and 0900/after 30-min rest	711.24 +/– 26.9 (SD) nM	560 +/– 65.2 (SD) nM	1.27	IL-6, TNF-α; plasma; between 0800 and 0900/after 30-min rest	None	IL-6—2.83 +/– 1.70 (SD) pg/ml; TNF-α—6.47 +/– 2.57 (SD) pg/ml	IL-6—1.75 +/– 1.1 (SD) pg/L; TNF-α - 5.40 +/– 1.5 (SD) pg/L
Landmann (36)	22 outpatients	22	Adult	No	No	Yes	No	Patient—clinical interview; control—not specified	Plasma; at 0800	505 +/– 27 (SEM) nM	465 +/– 35 (SD) nM	1.09	IFN-γ, TNF-α; plasma (IFN-γ); at 0800 (IFN-γ), *ex vivo* cells stimulated (TNF-α)	LPS	IFN-γ—30 +/– 8 (SEM) ng/L; TNF-α—1.42 +/– 0.4 (SEM) ng/L	IFN-γ—17 +/– 4 (SEM) ng/L; TNF-α—2.01 +/– 0.49 (SEM) ng/L
Lamers (37)	233 inpatients and outpatients	543	Adult	Yes—CAD (~5%); DM (~5%)	Yes—anxiety disorders	Yes	Yes (~5%)	Composite Diagnostic International Interview	Salivary; awakening response (area under curve to ground) at awakening, 30-, 45- and 60-min post-awakening/ variable waking time	19.38 +/– 4.41 (SD) nM	18.47 +/– 6.85 (SD) nM	1.05	CRP, IL-6, TNF-α; plasma; not specified	None	CRP—1.53 +/– 1.24 (SD) mg/L; IL-6—0.9 +/– 0.95 (SD) pg/ml; TNF-α – 0.91 +/– 0.96 (SD) pg/ml	CRP—1.12 +/– 3.23 (SD) mg/L; IL-6—0.73 +/– 2.58 (SD) pg/ml; TNF-α—0.84 +/– 1.90 (SD) pg/ml
Lisi (38)[b]	8	10	Adult	No	No	Yes	No	MINI	Salivary; at 0800	0.49 +/– 0.08 (SEM) μg/dL	0.43 +/– 0.08 (SEM) μg/dL	1.14	IL-1β, IL-6; mRNA from *ex vivo* cells stimulated	LPS	IL-1β—595.86 +/– 930.1 (SD) U; IL-6 — 1,322.65 +/– 1,740.07 (SD) U	IL-1β—300.37 +/– 442.48 (SD) U; IL-6—612.63 +/– 912.97 (SD) U
Lopes (39)	22 outpatients	15	Adult	No	No	Yes	No	SCID-IV	Salivary; at 0800/always prior to venipuncture	7.8 +/– 1.0 (SEM) nM	12.5 +/– 0.5 (SEM) nM	0.624	IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α; *ex vivo* cells stimulated	PHA	IL-2—512.14 +/– 109.12 (SEM) pg/ml; IL-4—346.37 +/– 87.48 (SEM) pg/ml; IL-6—3,931.82 +/– 880.15 (SEM) pg/ml; IL-10—1617.94 +/– 413.02 (SEM) pg/ml; IFN-γ—2,390.71 +/– 548.54; *n* = 22; TNF-α—2034.02 +/– 491.16 (SEM) pg/ml	IL-2—1060.90 +/– 189.40 (SEM) pg/ml; IL-4—2997.29 +/– 1,710.04 (SEM) pg/ml; IL-6—4,867.81 +/– 1,532.65 (SEM) pg/ml; IL-10—2,467 +/– 956.16 (SEM) pg/ml; IFN-γ—2,813.09 +/– 767.76; TNF-α— 2,063.64 +/– 593.13 (SEM) pg/ml

TABLE 1 | Continued

Study	Patients	Control	Age	Medical co-morbidity	Psychiatric co-morbidity	Medications in patients	Anti-inflammatories	Diagnostic method	Cortisol source; sample timing/ detail	Cortisol level patients	Cortisol level control	Cortisol ratio—patients/ control	Cytokine; source; sample timing/ detail	Stimulant	Cytokine level patient	Cytokine Level Control
Maes (40)	19 inpatients	10	Adult	No	No	Yes	No	Patient—SCID-III; control—not specified	Plasma; at 0800	18.92 +/– 4.32 (SD) μg/dL	21.65 +/– 10.10 (SD) μg/dL	0.87	IL-1β; *ex vivo* cells stimulated	PHA	2,225 +/– 1,773 (SD) pg/ml	1115 +/– 1105 (SD) pg/ml
Maes (41)	48 inpatients	32	Adult	No	No	Yes	No	Patient—SCID-IV; control—structured interview	Plasma; at 0900/after 30-min rest	9.7 +/– 4.5 (SEM) μg/dL	9.3 +/– 3.7 (SEM) μg/dL	0.96	IL-6, sIL-2R; plasma; at 0845/after 15-min rest	None	IL-6—3.5 +/– 0.3 (SEM) pg/ml; sIL-2R—293 +/– 69 (SEM) U/ml	IL-6—1.5 +/– 0.3 (SEM) pg/ml; sIL-2R—236 +/– 100 (SEM) U/ml
Maes (42)	17 inpatients	8	Adult	No	No	Yes	No	Patient—SCID-III; controls—not specified	Plasma; at 0800	19.34 +/– 4.53 (SD) μg/dL	22.7 +/– 10.6 (SD) μg/dL	0.85	IL-6; *ex vivo* cells stimulated	PHA	45.3 +/– 6.93 (SD) ng/ml	26.6 +/– 13.7 (SD) ng/ml
Marques-Deak (43)[a]	45–46 outpatients	36–39, matched	Adult	No	No	No	No	Patient—SCID-IV; control—not specified	Plasma; at 0800	11.6 +/– 3.8 (SD) μg/dL	12.4 +/– 5.5 (SD) μg/dL	0.94	IFN-γ, IL-1β, IL-6; plasma; at 0800	None	IFN-γ—197.4 +/– 230.8 (SD) IU/ml; IL-1β—36.4 +/– 18.5 (SD) ng/ml; IL-6—132.4 +/– 83.2 (SD) ng/ml	IFN-γ—148.4 +/– 149.8 (SD) IU/ml; IL-1β—35.2 +/– 14.1 (SD) ng/ml; IL-6—129.3 +/– 61.6 (SD) ng/ml
Martinac (44)	49 inpatients	40	Adult	No	No	No	No	Patient—MINI; control—not specified	Plasma; at 0800/after 30-min rest	748.6 +/– 419.31 (SD) nM	476 +/– 116.88 (SD) nM	1.57	CRP, IL-6, TNF-α; plasma; at 0800/after 30-min rest	None	CRP—1.4 +/– 0.84 (SD) mg/L; IL-6 - 2 +/– 0.38 (SD) pg/ml; TNF-α—5.9 +/– 2.29 (SD) pg/ml	CRP—0.7 +/– 0.31 (SD) mg/L; IL-6—1.0 +/– 0.77 (SD) pg/ml; TNF-α – 5.0 +/– 2.31 (SD) pg/ml
Nikkheslat (45)[b]	19–20 outpatients	27–33, matched	Geriatric (~68–70)	Yes—past MI (~40%); HTN (~75%); DM (~20%); dyslipidemia (~60%)	No	Yes (~40%)	No	Clinical Interview Schedule-Revised	Plasma; before 1000	CRP—288.80 +/– 119.29 (SD) nM; IL-6—290.79 +/– 123.91 (SD) nM	CRP—341.67 +/– 104.58 (SD) nM; IL-6—369.22 +/– 117.16 (SD) nM	CRP—0.85; IL-6—0.79	CRP, IL-6; plasma; before 1000	None	CRP—4.99 +/– 4.57 (SD) mg/L; IL-6—2.38 +/– 1.90 (SD) pg/ml	CRP—3.34 +/– 4.29 (SD) mg/L; IL-6—2.21 +/– 2.49 (SD) pg/ml
Rudzki (46)	34 outpatients	29	Adult	No	No	Yes	No	Clinical interview	Plasma; between 0800 and 0900	174.76 +/– 12.08 (SEM) μg/ml	136.35 +/– 10.29 (SEM) μg/ml	1.28	IL-1β, IL-6, TNF-α; plasma; between 0800 and 0900	None	IL-1β—0.122 +/– 0.14 (SEM) pg/ml; IL-6—2.07 +/– 2.58 (SEM) pg/ml; TNF-α—1.09 +/– 0.4 (SEM) pg/ml	IL-1β—0.43 +/– 0.26 (SEM) pg/ml; IL-6—1.26 +/– 0.1 (SEM) pg/ml; TNF-α—1.7 +/– 0.13 (SEM) pg/ml

TABLE 1 | Continued

Study	Patients	Control	Age	Medical co-morbidity	Psychiatric co-morbidity	Medications in patients	Anti-inflammatories	Diagnostic method	Cortisol source; sample timing/ detail	Cortisol level patients	Cortisol level control	Cortisol ratio—patients/ control	Cytokine; source; sample timing/ detail	Stimulant	Cytokine level patient	Cytokine Level Control
Simmons (47)[b]	26 outpatients	28	Adult	No	No	Yes	No	SCID-IV	Salivary; between 1900 and 2200	CRP—1.31 +/– 0.66 (SD) nM; IL-1RA—1.31 +/– 0.65 (SD) nM; IL-6—1.29 +/– 0.65 (SD) nM	CRP—1.21 +/– 0.52 (SD) nM; IL-1RA—1.20 +/– 0.51 (SD) nM; IL-6—1.20 +/– 0.56 (SD) nM	CRP - 1.085; IL-1RA - 1.091; IL-6 - 1.075	CRP, IL-1RA, IL-6; plasma; at 1200	None	CRP—3.17 +/– 2.91 (SD) mg/L; IL-1RA—0.35 +/– 0.20 (SD) ng/ml; IL-6—1.06 +/– 0.48 (SD) pg/ml	CRP—2.54 +/– 2.54 (SD) mg/L; IL-1RA—0.36 +/– 0.28 (SD) ng/ml; IL-6—0.72 +/– 0.36 (SD) pg/ml
Trzonkowski (48)[a]	10 inpatients	10, matched	Geriatric (~50–90)	Yes, multiple	Yes—MNCD (~50%)	No	Yes	SCID-IV	Plasma; between 0700 and 0800	355 +/– 35 (SD) nM	280 +/– 20 (SD) nM	1.27	IL-6, TNF-α; plasma; between 0700 and 0800	None	IL-6—650 +/– 140 (SD) fg/ml; TNF-α – 0.6 +/– 0.3 (SD) pg/ml	IL-6—230 +/– 20 (SD) fg/ml; TNF-α —0.3 +/– 0.05 (SD) pg/ml
Verduijn (49)[a]	1,083 outpatients	228	Adult	Yes, multiple	Yes—Substance use	Yes	Yes (~5%)	Composite Diagnostic International Interview	Salivary; awakening response (Area Under Curve to Ground) at awakening, 30-, 45- and 60-min post-awakening/ variable waking time	19.4 +/– 7.4 (SD) nM	18.2 +/– 7.0 (SD) nM	1.07	CRP, IL-6; plasma; around 0800	None	CRP—1.39 +/– 3.59 (SD) mg/L; IL-6—0.80 +/– 2.63 (SD) mg/L	CRP—1.14 +/– 3.09 (SD) mg/L; IL-6—0.71 +/– 2.47 (SD) mg/L
Weinstein (50)[b]	14 outpatients	14	Adult	No	No	Yes	No	SCID-IV	Plasma; between 1200 and 1600/after 30-min rest	IL-6/CRP—12.05 +/– 6.10 (SD) U; TNF-α—12.48 +/– 6.16 (SD) U	IL-6/CRP—11.71 +/– 5.38 (SD) U; TNF-α—12.05 +/– 5.41 (SD) U	IL-6/ CRP—1.029; TNF-α—1.036	CRP, IL-6, TNF-α; plasma; between 1200 and 1600/after 30-min rest	None	CRP—1.35 +/– 1.18 (SD) U; IL-6 - 3.0 +/–3.33 (SD) U; TNF-α—2.48 +/– 1.31 (SD) U	CRP—2.01 +/– 2.15 (SD) U; IL-6 - 1.23 +/– 1.13 (SD) U; TNF-α—3.11 +/– 1.83 (SD) U

Where only a single standardized interview is listed, it was applied to both patient and control. [a]cortisol and cytokine levels were not reported for every patient and control; analysis based on mean and standard deviation reported in paper. [b]analysis based on raw data provided by study authors. Abbreviations are as follows: CAD, coronary artery disease; DM, diabetes mellitus; MI, myocardial infarction; HTN, hypertension; MNCD, major neurocognitive disorder; SCID-IV, Structured Clinical Interview for DSM-IV; SCID-III, Structured Clinical Interview for DSM-III; MINI, Mini-international Psychiatric Interview; SD, standard deviation; SEM, standard error of the mean; PHA, phytohemagglutinin; ECP, eosinophil cationic protein; EOTAXIN-2, eosinophil chemotactic protein-2; IL-1RA, IL-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted.

IL-4 levels (25, 39), 23 studies reported IL-6 levels (13, 21, 22, 25, 28, 29, 32–34, 35, 37, 38, 41–49–50), one study reported IL-8 levels (22), three studies reported IL-10 levels (22, 25, 39), one study reported monocyte chemoattractant protein-1 (MCP-1) levels (25), one study reported regulated on activation, normal T cell expressed and secreted (RANTES) levels (27), one study reported soluble IL-2 Receptor (sIL-2R) levels (41), 15 studies reported TNF-α levels (24, 25, 27, 28, 32–36, 37, 39, 44, 46, 48, 50), and one study reported vascular endothelial growth factor (VEGF) levels (25).

As stated previously, we only meta-analyzed data for a specific cytokine if five or more studies reported values in patients and controls. We therefore meta-analyzed data for CRP, IFN-γ, IL-1β, IL-2, IL-6, TNF-α, comparing depressed patients with controls.

Irrespective of glucocorticoid resistance levels, patient levels of CRP (d = 0.23; 95% CI, −0.01 to 0.46), IFN-γ (d = 0.22; 95% CI, −0.02 to 0.47), IL-1β (d = 0.18; 95% CI, −0.24 to 0.61), and IL-2 (d = −0.12; 95% CI, −1.04 to 0.80) were all not significantly different from control. The number of studies included for these cytokines only just met the minimum number articulated above and in most cases the distribution of studies between "cortisolemic" states was uneven, leading to insufficient study number in each sub-group to conduct a formal analysis (data not shown).

IL-6 analysis was based on 1,850 patients and 1,232 controls. Overall effect size was 0.61 (95% CI, 0.36–0.85), demonstrating a significantly higher level of IL-6 in depressed patients than in controls (p < 0.0001; **Figure 2**). Heterogeneity was visually evident in this analysis, and this was reflected in statistical analysis of the same (τ^2 = 0.25; p < 0.00001; I^2 = 84%). Overall effect size was insensitive to serial exclusion of studies.

Grouping studies by glucocorticoid resistance revealed that when glucocorticoid resistance in patients was higher in either the hypercortisolemic or eucortisolemic sub-groups (ratio patient:control > 1.2, or 0.8 < ratio patient:control < 1.2, respectively), overall effect size was significantly larger than in the two studies where patients were hypocortisolemic (ratio patient:control < 0.8) (**Figure 2**). Notably, hypercortisolemic patients (d = 0.94; 95% CI, 0.29–1.59) tended to produce more IL-6 compared with controls than did eucortisolemic patients (d = 0.52; 95% CI, 0.26–0.77), but this difference was not

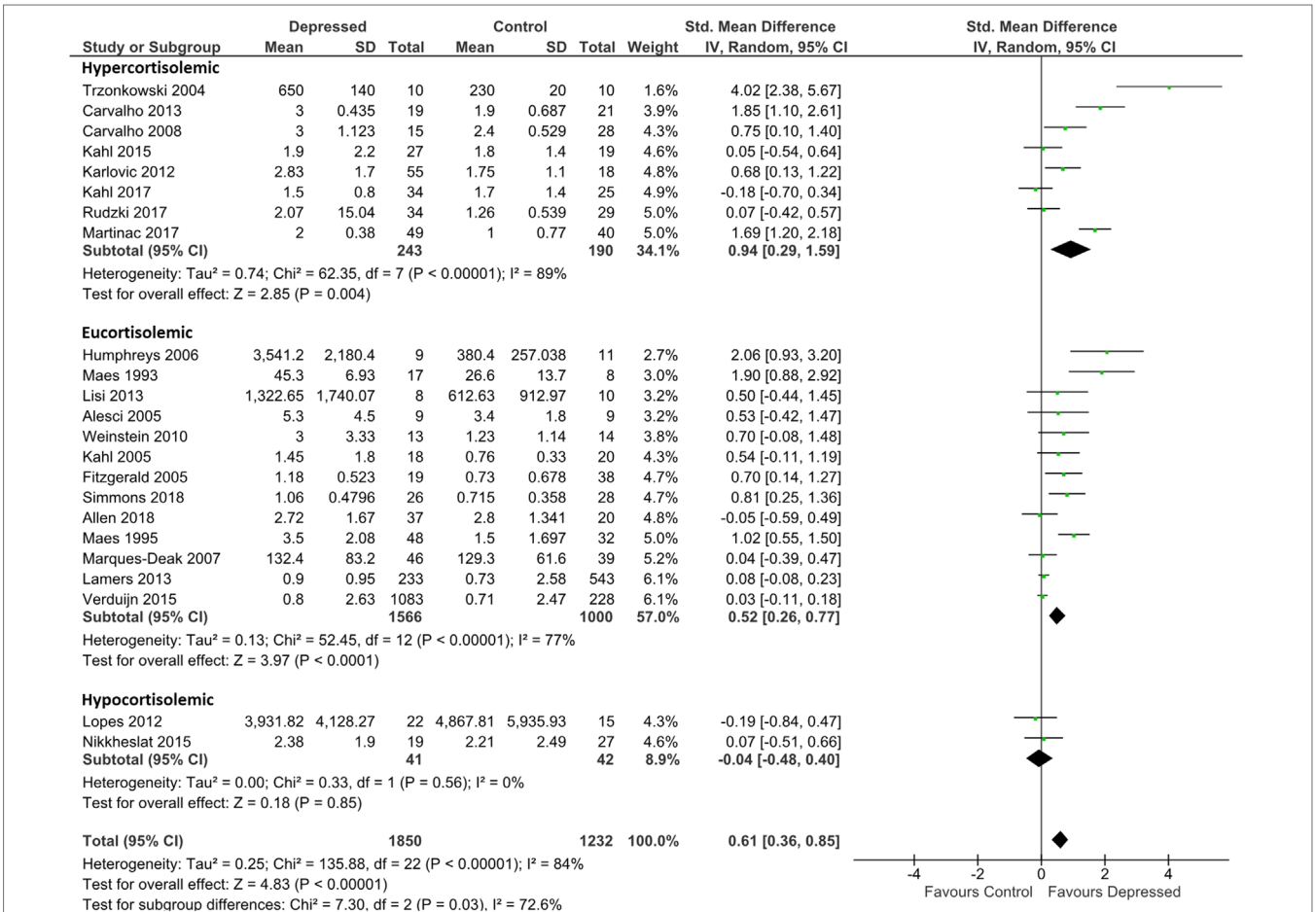

FIGURE 2 | Forest plot analysing effect size for IL-6, sub-grouping studies by relative plasma cortisol levels. Hypercortisolemic corresponds to patient:control plasma cortisol ratio > 1.2; eucortisolemic corresponds to patient:control plasma cortisol ratio > 0.8 or < 1.2; hypocortisolemic corresponds to patient:control plasma cortisol ratio < 0.8.

statistically significant (**Figure 2**). The difference in effect size observed between hypercortisolemic and eucortisolemic sub-groups was insensitive to serial exclusion of studies. Overall, sub-group difference testing revealed a significant result ($\chi^2 = 7.3$; $df = 2$; $p = 0.03$), but we did not interpret this to signify the existence of a true difference between the three sub-groups as the 95% confidence intervals associated with effect sizes in each sub-group sequentially overlapped as one moved from hypocortisolemic to eucortisolemic to hypercortisolemic.

Sub-grouping by presumed glucocorticoid resistance status did not appreciably reduce heterogeneity of the meta-analysis in the hypercortisolemic ($\tau^2 = 0.74$; $p < 0.00001$; $I^2 = 89\%$) or the eucortisolemic ($\tau^2 = 0.13$; $p < 0.00001$; $I^2 = 77\%$) sub-groups. The hypocortisolemic sub-group was homogeneous visually and statistically (**Figure 2**).

Analysis for TNF-α was based on data from 604 patients and 864 controls. Irrespective of presumed glucocorticoid resistance status, overall effect was moderate [$d = 0.40$; 95% CI, 0.12–0.68); $p = 0.006$]. Heterogeneity was visible on forest plots (**Figure 3**) and was reflected in measures of heterogeneity in the overall analysis ($\tau^2 = 0.21$; $p < 0.00001$; $I^2 = 78\%$). Subgrouping by glucocorticoid resistance status revealed a non-significant trend for hypercortisolemic patients to produce more TNF-α than eucortisolemic patients when compared with control ($d = 0.46$; 95% CI, 0.12–0.79 and $d = 0.39$; 95% CI, −0.19 to 0.98, respectively). An insufficient number of studies reported TNF-α levels in hypocortisolemic patients to allow comparison of this sub-group to the hypercortisolemic and eucortisolemic sub-groups (**Figure 3**). Significant heterogeneity was evident in both latter sub-groups ($\tau^2 = 0.15$; $p < 0.005$; $I^2 = 65\%$ and $\tau^2 = 0.43$; $p < 0.00001$; $I^2 = 86\%$, respectively).

Censoring of Kahl et al. (34), which was a visual outlier in the eucortisolemic sub-group (**Figure 3**) accentuated the difference in effect size between the hypercortisolemic and eucortisolemic sub-groups (whole data set $d = 0.46$ and 0.39 versus adjusted $d = 0.46$ and 0.07, respectively), but this did not result in a statistically significant difference between the two sub-groups. Eucortisolemic sub-group heterogeneity significantly decreased with this censure (adjusted $\tau^2 = 0.03$; $p = 0.18$; $I^2 = 36\%$). Serial exclusion of Rudzki et al. (46) and Trzonkowski et al. (48) from the hypercortisolemic sub-group did not significantly alter the difference in effect size between the hypercortisolemic and the eucortisolemic sub-groups. Only exclusion of Rudzki et al. (46) from the hypercortisolemic sub-group significantly reduced sub-group heterogeneity (adjusted $\tau^2 = 0.06$; $p = 0.10$; $I^2 = 43\%$).

ii) Glucocorticoid Resistance Measured by DST or Other Endocrine Suppression Test in Patients and Controls

Six hundred forty articles were identified in our database search (**Figure 1**). After removal of duplicates and review of titles and abstracts to ensure that studies met our inclusion

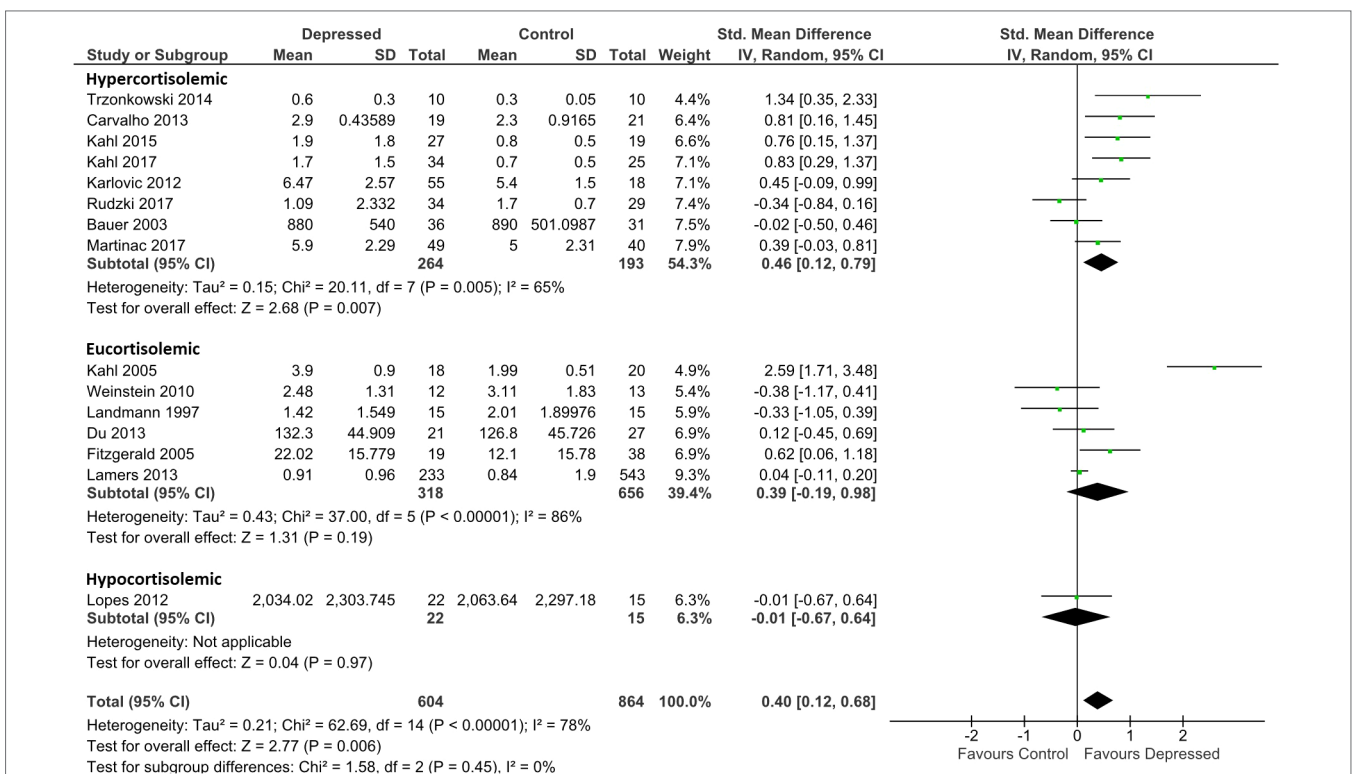

FIGURE 3 | Forest plot analysing effect size for TNF-α, sub-grouping studies by relative plasma cortisol levels. Hypercortisolemic corresponds to patient:control plasma cortisol ratio > 1.2; eucortisolemic corresponds to patient:control plasma cortisol ratio > 0.8 or < 1,2; hypocortisolemic corresponds to patient:control plasma cortisol ratio < 0.8.

criteria, 20 articles were retrieved for full-text review. 11 studies were excluded for the following reasons: four were conference abstracts for which we could not obtain associated study data; four studies did not include a control group; two studies did not use an independent measure of glucocorticoid resistance; one study did not measure glucocorticoid resistance. This left nine studies for our review (**Table 2**). All included studies were of a similar design to that described in the section Glucocorticoid Resistance as Assessed by Relative Plasma Cortisol Levels Between Depressed Patients and Controls.

Eight of nine studies evaluated glucocorticoid resistance using the DST. One study (28) used a previously validated cutaneous glucocorticoid resistance test (53) to evaluate glucocorticoid resistance. For this study, we used equivalent suppressor counts in patients and controls to calculate the "glucocorticoid resistance index." Six studies reported unstimulated levels of cytokines, either measured by ELISA or by mRNA expression levels of cytokines (13, 28, 29, 36, 51, 52). Six studies used stimulation with mitogens to elicit increased cytokine secretion either from whole blood or *in vitro* culture of immune cells (13, 24, 29, 36, 38, 40), but only three studies relied exclusively on this method (24, 38, 40).

All studies were completed on adult patients and controls who had no medical nor psychiatric co-morbidities, including substance use. Seven of nine studies reported data from patients who were at least partially treated with antidepressants at the time of assay. No subjects in any of the studies were using anti-inflammatory medications or had a history or current manifestation of inflammatory illnesses.

One study reported IFN-γ levels (36), 2 studies reported IL-1β levels (38, 40), one study reported IL-2 levels (24), six studies reported IL-6 levels (13, 28, 29, 38, 51, 52), and three studies reported TNF-α levels (24, 28, 36). Five studies examined multiple cytokines (24, 28, 29, 36, 38), though not all studies reported a level for each cytokine examined (29).

IL-6 levels from studies that examined this cytokine were used to determine effect sizes, whereas in the case of studies that did not examine IL-6, unstimulated levels of another cytokine were used (see **Figure 4**). This approach was taken to minimize heterogeneity introduced by pooling plasma and stimulated study results. All patients were at least as glucocorticoid resistant as controls.

When studies were ranked from high glucocorticoid resistance in patients to low glucocorticoid resistance using the "glucocorticoid resistance index," there was a slight trend for studies that reported high glucocorticoid resistance in patients to produce larger effect sizes (based on 170 patients and 187 controls; **Figure 4A**). Examination of regression residuals did not support the existence of a significant trend, however (data not shown). Patients overall produced significantly higher levels of cytokines than did controls (d = 0.81; 95% CI, 0.39–1.23); p = 0.0002). Significant heterogeneity was noted in this analysis (τ^2 = 0.27; p = 0.001; I^2 = 69%).

Since three of nine studies used in the above analysis only were able to contribute data obtained by stimulating *in vitro* cultured immune cells with mitogens, we hypothesized that this experimental dichotomy may have introduced heterogeneity that affected our overall analysis.

When we performed moderator analysis that separated studies into those where cytokine levels were obtained from plasma and those where cytokine values were obtained from *in vitro* stimulated immune cells, there was a reduction in heterogeneity within subgroups (plasma: τ^2 = 0.20; p = 0.02; I^2 = 62%; stimulated: τ^2 = 0.03; p = 0.31; I^2 = 15%; **Figure 4B**). Furthermore, this analysis revealed the preservation of effect size in the plasma sub-group (d = 1.04; 95% CI, 0.57–1.50) and separation of this effect from that seen in the *in vitro* stimulated sub-group (d = 0.24; 95% CI, −0.20 to 0.67). the difference between these two sub-groups was statistically significant (χ^2 = 6.07; df = 1; p = 0.01).

iii) Glucocorticoid Resistance Measured in *In Vitro* or from *GR* Expression Levels in Patients and Controls

Two hundred sixty-five articles were identified in our database search (**Figure 1**). After removal of duplicates and review of titles and abstracts to ensure that studies met our inclusion criteria, five articles were retrieved for full-text review. One study was excluded since it assessed only depressive symptoms and did not assess for the presence of a major depressive episode using a standardized clinical/diagnostic interview. One study was excluded because it assessed only children aged 6 to 11 years. Through expert consultation during peer review, we identified one additional study that did not appear in our literature search. This left four studies for our review (**Table 3**). All studies were of a case–control design.

Two of four studies used suppression of lipopolysaccharide (LPS)-induced IL-6 production by exogenous glucocorticoids to measure glucocorticoid resistance (13, 45, 54). Two studies used *GR* expression to assess glucocorticoid resistance (55, 56). Patients in all studies generally displayed at least as much glucocorticoid resistance as controls did (**Table 3**).

Three of four studies examined adult patients and controls with no medical or psychiatric co-morbidities. The remaining study (45) examined geriatric patients only (age range 68–70). This latter study was conducted in subjects already known to suffer from coronary artery disease and thus both patients and controls displayed several cardiac co-morbidities. These included hypertension (~75% prevalence), dyslipidemia (~60% prevalence), diabetes (~20% prevalence), and previous myocardial infarction (~40% prevalence), although the prevalence of these co-morbidities were relatively equal between patients and controls.

The prevalence of patient antidepressant usage in each study varied between ~40% and 100%. None of the reviewed studies included patients or controls who were taking anti-inflammatory medications.

The most commonly reported cytokine level was IL-6, which was reported by all studies (13, 45, 54–56). IL-6 levels were used from three studies (13, 55, 56) to determine effect size, whereas CRP was used for the other study (45).

Overall effect size, based on 147 patients and 118 controls, was significantly different from 0 (d = 1.35; 95% CI, 0.53–2.18;

TABLE 2 | Studies included in analysis using endocrine suppression tests to measure relative glucocorticoid resistance.

Study	Patients	Controls	Medications used in patients?	Diagnostic method	Cortisol source	Endocrine suppression test used	Endocrine suppression test results	Glucocorticoid resistance index	Cytokine and source	Stimulant	Cytokine patient	Cytokine control
Bauer (24)	36 inpatients	31	Yes	Clinical interview	Salivary	DST	Patient—26/36; control—30/31 suppressors	0.25	IL-2; TNF-α; *ex vivo* cells stimulated	PHA (IL-2), LPS (TNF-α)	IL-2—338.5 +/– 69.8 (SEM) pg/ml; TNF-α—870 +/– 115 (SEM) pg/ml	IL-2—297.1 +/– 101.7 (SEM) pg/ml; TNF-α—880 +/– 100 (SEM) pg/ml
Carvalho (13)	15 inpatients	28	Yes	Patient—SCID-IV; control—not specified	Plasma	DST	Patient—0/15 suppressors; control—28/28 suppressors	1	IL-6; plasma and whole blood stimulated	LPS	Plasma—3.0 +/– 0.29 (SEM) pg/ml; whole blood stimulated—1,025 +/– 175 (SEM) pg/mL	Plasma—2.4 +/– 0.1 (SEM) pg/ml; whole blood stimulated - 875 +/– 150 (SEM) pg/ml
Fitzgerald (28)	19	38	Yes	Patient—clinical interview; control—not specified	Plasma	Skin blanching secondary to topical corticosteroid cream	Patient—0/19 suppressors; control—38/38 suppressors	1	IL-6, TNF-α; plasma	None	IL-6—1.18 +/– 0.12 (SEM) pg/ml; TNF-α—22.02 +/– 3.62 (SEM) pg/ml	IL-6—0.73 +/– 0.11 (SEM) pg/ml; TNF-α—12.10 +/– 2.56 (SEM) pg/ml
Humphreys (29)	9	11	No	Patient—SCID-IV; control—not specified	Plasma	DST	Patient—7/9 suppressors; control—10/11 suppressors	0.14	IL-6; *ex vivo* cells unstimulated and stimulated	LPS	Unstimulated—3,541.2 +/– 726.8 (SEM) pg/ml; stimulated—19,867.7 +/– 3649.2 (SEM) pg/ml	Unstimulated—380.4 +/– 77.5 (SEM) pg/ml; stimulated—33,142.2 +/– 1,547.2 (SEM) pg/ml
Landmann (36)	22 outpatients	22	Yes	Patient—clinical interview; control—not specified	Plasma	DST	Patient—21/22 suppressors; control—21/22 suppressors	0	IFN-γ, TNF-α; plasma (IFN-γ) and *ex vivo* cells stimulated (TNF-α)	LPS	IFN-γ—30 +/– 8 (SEM) ng/L; TNF-α—1.42 +/– 0.4 (SEM) ng/L	IFN-γ—17 +/– 4 (SEM) ng/L; TNF-α - 2.01 +/– 0.49 (SEM) ng/L
Lisi (38)	8	10	Yes	MINI	Salivary	DST	IL-1β—patient—5/8 suppressors, control—4/6 suppressors; IL-6—patient—6/8 suppressors, control—4/7 suppressors	IL-1β—0.0625; IL-6 ——0.31	IL-1β, IL-6; mRNA from *ex vivo* cells stimulated	LPS	IL-1β—595.86 +/– 930.1 (SD) U; IL-6 – 1322.65 +/– 1740.07 (SD) U	IL-1β—444.68 +/– 488.03 (SD) U; IL-6—695.3 +/– 1,027.38 (SD) U
Maes (40)	19 inpatients	10	Yes	Patient—SCID-III; control—not specified	Plasma	DST	Patient—13/19 suppressors; control—8/10 suppressors	0.145	IL-1β; *ex vivo* cells stimulated	PHA	2,225 +/– 1,773 (SD) pg/ml	1,115 +/– 1,105 (SD) pg/ml
Musselman (51)	11 inpatients and outpatients	9	No	SCID-III	Plasma	DST	Patient—8/11 suppressors; control—9/9 suppressors	0.27	IL-6; plasma	None	172.5 +/– 180.42 (SD) pg/ml	20.05 +/– 25.86 (SD) pg/ml
Soygur (52)	30 inpatients	30	Yes	SCID-IV	Plasma	DST	Patient—63% suppressors; control—100% suppressors	0.37	IL-6; plasma	None	17.75 +/– 5.15 (SD) ng/ml	9.5 +/– 4.66 (SD) ng/ml

Where only a single standardized interview is listed, it was applied to both patient and control. Abbreviations are as for **Table 1**, *with the following exception: DST, dexamethasone suppression test.*

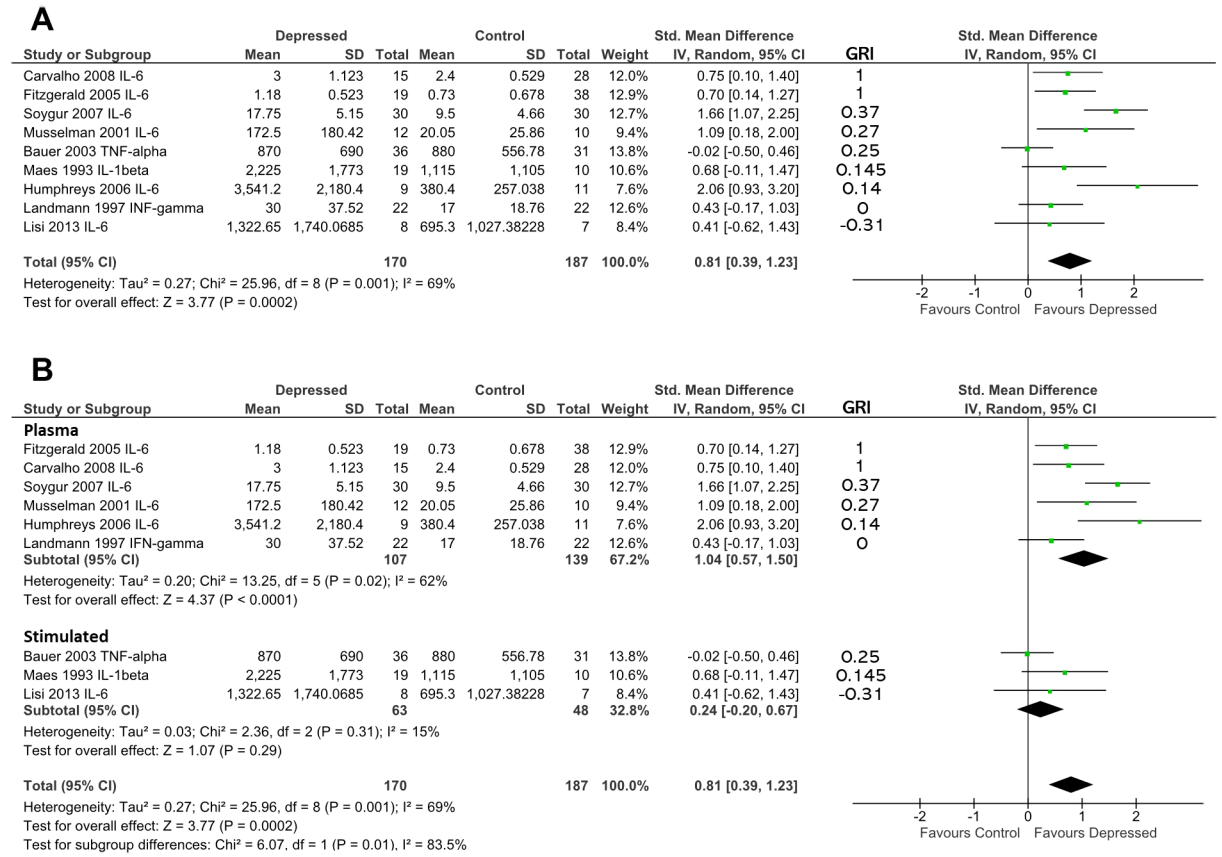

FIGURE 4 | (A) Forest plot reporting effect size for cytokine stratified by "glucocorticoid resistance index" (GRI) calculated from DST or other endocrine suppression test. The specific cytokine selected for inclusion (based on criteria enumerated in Methods) is listed after the study name. Studies are ranked by GRI from high to low. **(B)** Forest plot of studies in **(A)** sub-grouped by source of cytokine. Stimulated levels were obtained from *in vitro* cultured cells using the stimulant specified in **Table 2**. Rank based on GRI (listed) as in **(A)**.

p = 0.001; **Figure 5**). Heterogeneity was high (τ^2 = 0.61; p < 0.0001; I^2 = 87%). Sensitivity analysis demonstrated that the overall effect size was mildly sensitive to exclusion only of Carvalho et al. (55) (adjusted effect size following study removal of d = 1.26; 95% CI, −0.01 to 2.53). Ranking studies by calculated glucocorticoid resistance did not reveal an obvious positive association between glucocorticoid resistance and cytokine production (**Figure 5**).

Building on our ability to standardize measurements of glucocorticoid resistance obtained through DST or other endocrine suppression tests, *in vitro* assays of GR function and *GR* expression levels using our glucocorticoid resistance index, we conducted a combined analysis of the studies included in **Figure 4** and **5**, using the effect sizes and glucocorticoid resistance index values reported in those figures (**Figure 6**). The data presented for Carvalho et al. (13) in **Figure 4A** was used for this analysis as inclusion of the data for this study from **Figure 5** as well would have resulted in duplication bias. For 302 patients and 277 controls, overall effect size was moderate (d = 1.02; 95% CI, 0.55–1.49; p < 0.0001], but heterogeneity was high (τ^2 = 0.56; p < 0.00001; I^2 = 84%). Ranking studies from high to low glucocorticoid resistance index did not reveal a significant trend for higher inflammation to be associated with higher levels of glucocorticoid resistance in patients compared with controls (**Figure 6**). This was confirmed by observation of regression residuals (data not shown).

DISCUSSION

Individual studies have reported a possible positive association between glucocorticoid resistance and cytokine-mediated inflammation in depression. Other studies have failed to find evidence of the same phenomenon. The conflicting nature of the primary literature, combined with a consensus in the field that such a positive association indeed exists (11, 57), lead us to conduct the systematic review and meta-analytic work described above.

Initially we hypothesized that we would validate a positive association between glucocorticoid resistance and cytokine-mediated inflammation. Despite multiple methods of examining our hypothesis, however, we found only modest evidence to support this idea, with the largest effects noticeable when glucocorticoid resistance is measured by plasma or salivary cortisol. Furthermore, when we combined data in which glucocorticoid resistance was

TABLE 3 | Studies included in analysis using *in vitro* assays of GR function or *GR* expression to measure relative glucocorticoid resistance.

Study	Patients	Control	Age	Medical co-morbidity	Medications in patients	Diagnostic method	*GR* expression patients	*GR* expression control	*In vitro* assay % of basal with glucocorticoid patients	*In vitro* assay % of basal with glucocorticoid controls	Glucocorticoid resistance index	Cytokine measured	Cytokine level patient	Cytokine level control
Carvalho (13, 54)	15 inpatients	28	Adult	No	Yes	Patient—SCID-IV; control—not specified			77 +/– 7 (SEM) %	56 +/– 13 (SEM) %	0.25	IL-6; plasma	1025 +/– 175 (SEM) pg/ml	875 +/– 150 (SEM) pg/ml
Carvalho (55)[a]	47 inpatients	42	Adult	No	Yes	Patients—SCID-IV; control—clinical interview	0.8	1			0.2	IL-1β, IL-6, IL-8; plasma	IL-1β—58.75 +/– 43.93 (SD) pg/ml; IL-6—1.525 +/– 1.104 (SD) pg/ml; IL-8—55 +/– 40.55 (SD) pg/ml	IL-1β—22.5 +/– 13.79 (SD) pg/ml; IL-6 —0.2625 +/– 0.08 (SD) pg/ml; IL-8—22.5 +/– 18.39 (SD) pg/ml
Cattaneo (56)	74 outpatients	34	Adult	Not specifically excluded	Yes	Patient—Schedules for Clinical Assessment in Neuropsychiatry; control—Psychosis Screening Questionnaire	0.85 +/– 0.01 (SEM)	1.03 +/– 0.02 (SEM)			0.17	IL-1A, IL-1B, IL-4, IL-6, IL-7, IL-8, IL-10, MIF, TNF; whole blood	IL-1α—1.00 +/– 0.02 (SEM); IL-1β—1.51 +/– 0.03 (SEM); IL-4—0.90 +/– 0.02 (SEM); IL-6—1.32 +/– 0.01 (SEM); IL-7—0.99 +/– 0.02 (SEM); IL-8—1.01 +/– 0.01 (SEM); IL-10—1.02 +/– 0.01 (SEM); MIF—1.30 +/–0.03 (SEM); TNF—1.55 +/– 0.04	IL-1α—0.96 +/– 0.04 (SEM); IL-1β—1.03 +/– 0.03 (SEM); IL-4—0.99 +/– 0.02 (SEM); IL-6—1.08 +/– 0.02 (SEM); IL-7—1.03 +/– 0.05 (SEM); IL-8—1.00 +/– 0.04 (SEM); IL-10—1.00 +/– 0.02 (SEM); MIF—0.98 +/– 0.04 (SEM); TNF—0.97 +/– 0.04 (SEM)

TABLE 3 | Continued

Study	Patients	Control	Age	Medical co-morbidity	Medications in patients	Diagnostic method	*GR* expression patients	*GR* expression control	*In vitro* assay % of basal with glucocorticoid patients	*In vitro* assay % of basal with glucocorticoid controls	Glucocorticoid resistance index	Cytokine measured	Cytokine level patient	Cytokine level control
Nikkheslat (45)[b]	11 outpatients	14, matched	Geriatric (~68-70)	Yes – past MI (~40%); HTN (~75%); DM (~20%); Dyslipidemia (~60%)	Yes (~40%)	Clinical Interview Schedule – Revised			63.05 +/− 20.57 (SD) %	63.63 +/− 15.13 (SD) %	-0.016	CRP, IL-6; plasma	CRP—6.24 +/− 4.03 (SD) mg/L; IL-6—1.82 +/− 1.43 (SD) pg/ml	CRP—3.79 +/− 4.83 (SD) mg/L; IL-6—2.55 +/− 2.49 (SD) pg/ml

Where only a single standardized interview is listed, it was applied to both patient and control. [a]*identified during peer review process.* [b]*analysis based on raw data provided by study authors. Abbreviations are as for* **Tables 1** *and* **2**, *with the following exception: MIF, macrophage migration inhibitory factor.*

measured by the DST or other endocrine suppression tests, *in vitro* assays of GR function and *GR* expression, we were unable to increase the resolution of our analysis (**Figure 6**). The cross-sectional nature of our analysis may have obscured a significant mild-to-moderate trend in the relationship between inflammation and more systemic measures of glucocorticoid resistance (e.g., cortisol levels, DST) that would have otherwise been detected by prospective studies specifically designed to address this question. A limitation of the current analysis is the design of the included studies, but in the absence of further published studies, we feel that our work is unlikely to have missed a significant trend.

We took an unbiased and inclusive approach to our literature search and review methodology. Although this may have contributed to the increased heterogeneity of meta-analytic results observed, it also allowed as many possible manifestations of glucocorticoid resistance to be included. At the same time, each measure of glucocorticoid resistance that we invoked had its limitations. Serum cortisol levels are not always elevated in depressed patients, as demonstrated by our review [e.g., Refs. (39) and (45)] and by other studies that were outside of our inclusion criteria (58–60). Work in the elderly suggests that frailty drives exhaustion of the HPA axis in depression, possibly leading to hypocortisolemia (59, 61, 62) whereas chronic over-stimulation of the HPA axis in other age groups can cause long-term hypocortisolemia (61, 63). Furthermore, diurnal variations in cortisol levels are a well-known phenomenon of the HPA axis, with the highest cortisol levels evident in healthy subjects within the first hour of awakening from sleep. Most studies incorporated in our analysis (94%) measured cortisol in the morning hours, but not all targeted the hour following arousal (see **Table 1**). In fact, the hours of cortisol measurement spanned 0700–1100. This could have created variability in our assessment of glucocorticoid resistance in a given study, perhaps hampering the aggregation of data reported in **Figures 2** and **3**. On the other hand, the fact that most studies in our analysis restricted their measurement of cortisol to morning hours suggests that any variability that may have been introduced by this factor is likely minimal. Along the same line, levels of cytokines are also known to cycle in a circadian fashion. For example, the nadir of IL-6 in healthy subjects occurs between the hours of 0800 and 1000 (64), very near to the hours that cortisol experiences its zenith. In the analysis represented in **Figures 2** and **3**, almost all studies (96%) that reported plasma levels of cytokines collected samples concurrently with those used to assess cortisol levels. The adherence of most studies to rigid timing when measuring cortisol and cytokines means that our assessment of the impact of glucocorticoid resistance on inflammation using reported cortisol levels is unlikely to have been significantly affected by circadian variabilities in these two factors.

Inflammation associated with chronic medical conditions can also compound variabilities between the included studies (65). We invoked the DST as a measure of glucocorticoid resistance to circumvent some of these limitations, but by its nature the DST measures peripheral glucocorticoid resistance only. Challenges persist in translating findings from the DST into inferences about effects in the central nervous system (CNS) and DST results are subject to a number of confounders such co-morbid medical

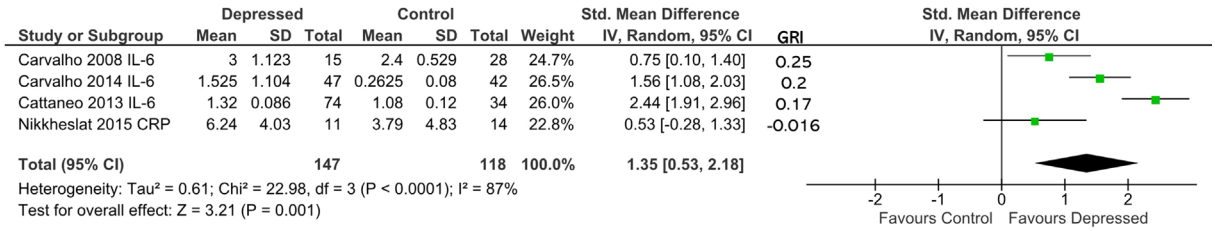

FIGURE 5 | Forest plot reporting effect size for cytokine stratified by "glucocorticoid resistance index" (GRI) calculated from *in vitro* GR functional assay or GR expression. The specific cytokine selected for inclusion (based on criteria enumerated in Methods) is listed after the study name. Studies are ranked by GRI from high to low.

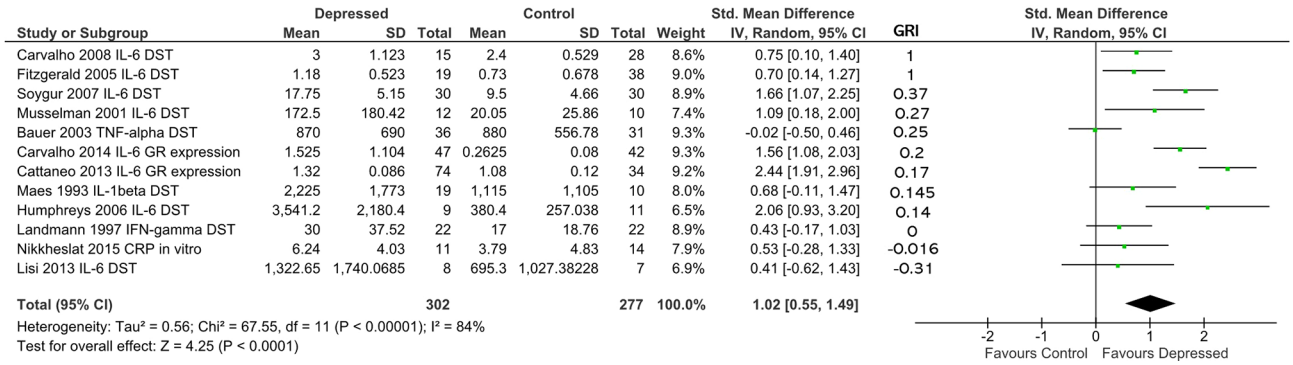

FIGURE 6 | Forest plot reporting effect size for cytokine stratified by "glucocorticoid resistance index" (GRI) calculated from DST or other endocrine suppression test results, *in vitro* GR functional assay or *GR* expression. Studies are ranked by GRI from high to low. All study data included in this figure were previously shown in **Figures 4** or **5**. Only the data for Carvalho et al. (13) from **Figure 4A** was used in this analysis as inclusion of data from this study from both **Figure 4A** and **Figure 5** would have introduced duplication bias. Listed beside each study is the glucocorticoid resistance outcome measure that was used to calculate the GRI.

illnesses (66). Finally, *in vitro* measurements of glucocorticoid resistance represent the most controlled mechanism through which to test peripheral resistance to glucocorticoids but suffer, like the DST, from limitations in translating their findings to the CNS. *GR* mRNA expression levels suffer from the same limitation (17).

Combining all three of these measures of glucocorticoid resistance has allowed us to take a complementary approach in our analysis, maximizing the likelihood that any trends present would be detected. We found that studies that used *in vitro* measures of GR function and/or *GR* expression to measure glucocorticoid resistance delivered the largest aggregate effect size [d = 1.35; 95% CI (0.53, 2.18)], but this must be balanced against the observation that we did not detect an obvious positive association between glucocorticoid resistance and cytokine production in that analysis. Thus, it is tempting to speculate that *in vitro* measurement of GR function and/or *GR* expression may be more likely to detect a significant effect, perhaps by removing potential confounders from the analysis. Verification of this contention will require further studies using these measures to be conducted. The other two approaches used to assess glucocorticoid resistance in our study only delivered effect sizes between 0.5 and 1. These measures of glucocorticoid resistance may be subject to greater confounding. In the case of relative cortisol levels as a measurement of glucocorticoid resistance, studies included in our analysis obtained cortisol from varying sources (e.g., plasma and salivary) and the timing of cortisol measurement may have varied slightly between studies, even when a standardized time of collection was reported. Both possibilities could have created small variations between studies. In the case of the DST or other endocrine suppression tests as a measure of glucocorticoid resistance, a significant source of variation could be the different cut-off levels used by the included studies to classify a participant as a non-suppressor. As we mentioned in our Methods, all cut-offs used exceeded the generally accepted value of 1.8 μg/dL (17), but we were unable to create a standardized cut-off level as we did not possess the raw post-dexamethasone cortisol values that would have allow us to make such a determination. Taken together, we contend that although all measurements of glucocorticoid resistance are subject to confounding, *in vitro* measurements of GR function and/or *GR* expression may represent the method least likely to be subject to bias as *in vitro* assays allow tighter control of potential confounders and *GR* expression may represent a more durable measure of the effects of excess glucocorticoids in depressed patients than do cross-sectional measurements of cortisol or cortisol responses.

In this regard, using *GR* expression to identify glucocorticoid resistance in depression is a relatively new technique.

Matsubara et al. (67) were among the first to identify decreased expression of the α transcript of the GR in depressed patients (*GRα* mRNA encodes the active form of the GR). Prior to this it was believed that the alterations in GR function in depression were driven primarily by post-translational modifications to the GR or its signalling pathways (68). Two subsequent studies (55, 56), including one from our group (56), confirmed that *GR* expression is indeed reduced in depressed patients. Carvalho et al. (55) found that increased levels of IL-8 in depressed patients correlated inversely with *GR* expression levels, suggesting that a certain level of intrinsic inflammation may occur independently of glucocorticoid resistance in depression, and that this inflammation plays a key role in the subsequent development of glucocorticoid resistance and further immune dysregulation through downregulation of *GR* transcript expression. This compounds the many impairments in GR function that occur during glucocorticoid resistance, including impaired translocation of the active GR from the cytoplasm to the nucleus, reduced affinity of the GR for its transcriptional binding sites and activation of competitor isoforms of the GR that impair the function of GRα (3). Although full exploration of the mechanism that underlies decreased *GR* expression in depression due to glucocorticoid resistance is still to come, the importance of *GR* expression as an independent signifier of glucocorticoid resistance is reinforced by the observation that *GR* expression levels did not correlate with the results of the DST in the population of depressed patients studied by Matsubara et al. (67). Furthermore, a reversal of decreased *GR* expression is seen when patients are treated with antidepressants (56), arguing that *GR* expression represents an important outcome of glucocorticoid resistance. Including *GR* expression analysis along with the DST and cortisol studies in any characterization of glucocorticoid resistance in depression is therefore highly desirable.

Our development of the glucocorticoid resistance index, a normalized way to compare glucocorticoid resistance between patients and controls across multiple studies, is rooted in the concept of relative differences. Thus, small absolute differences in resistance between patients and controls in a given study could result in a large relative difference. We feel that this limitation is acceptable as it is challenging to compare studies that report absolute counts of suppressors and non-suppressors in the DST or other *in vitro* measures of glucocorticoid resistance. Our index allows rapid conversion of absolute measures of glucocorticoid resistance into meaningful differences through which studies can be compared. As well, our index allows the conversion of count data on glucocorticoid resistance into a continuous measure of the same, possibly facilitating the use of meta-regression techniques in moderator analysis. In this study, we considered the use of meta-regression on our moderator, but preliminary analysis demonstrated no significant trends in plots of regression residuals (see Results). Nonetheless, we were able to capitalize on the power of the glucocorticoid resistance index when we combined data from studies using the DST or endocrine suppression tests, *in vitro* assays of GR function and *GR* expression in **Figure 6**.

Ideally, a common measure of glucocorticoid resistance would be used. The field as a whole has faced challenges in this task as it is unclear which measure should be adopted as standard (17). This likely relates in part to the variable results that are obtained when multiple measurements of resistance are applied to a single population of patients and controls. For example, when we used the cortisol levels of patients and controls, as well as *in vitro* measures of GR function to assess glucocorticoid resistance in the subjects analyzed by Nikkheslat et al. (45), we noted variances in the degree of glucocorticoid resistance inferred. We do not feel that this represents a systemic flaw in the data reported; rather, it is likely the result of the variability found when glucocorticoid resistance is quantified by different measures. We propose that multiple methods for evaluating glucocorticoid resistance are likely ideal for comparing data across many studies. Studies, such as those conducted by Miller et al. (69), which although not included in our meta-analysis due to conflict with our inclusion criteria, represent an ideal approach, assessing glucocorticoid resistance and inflammation through multiple independent measures.

Several studies that measured glucocorticoid resistance and cytokine production were identified by our review but were not be included in our meta-analyses. Doolin et al. (70) examined the relationship between waking salivary cortisol levels and mRNA expression of *IL-1β* and *IFN-γ* in whole blood. Measurements of both cytokine mRNA levels were non-Gaussian, with outcomes reported only as the results of non-parametric tests. We were unable to obtain raw data to circumvent this limitation. Nonetheless, Doolin et al. (70) report an inverse association between decreased morning cortisol reactivity, a marker of glucocorticoid resistance in depression, and *IL-1β* expression. No such relationship between cortisol reactivity and *IFN-γ* expression was found. Therefore, some support for an association between glucocorticoid resistance increased cytokine production in depression was found.

Stelzhammer et al. (71) used multiplex immunoassay and mass spectrometry to examine the relationship between plasma cortisol and IL-1RA, IL-16, and MIF in depressed and controls. They report higher levels of plasma cortisol and all three inflammatory markers in depressed patients but did not report parametric outcomes of statistical tests. Therefore, we were unable to incorporate this data into our analysis, but observe that elevated plasma cortisol and cytokine production co-existed in depressed patients (71), consistent with models promulgated in the literature (11).

Finally, Maes et al. (72) utilized the same patients as those in Maes et al. (40), undertaking *in vitro* analysis of both glucocorticoid resistance and cytokine production in cell culture supernatants. A strong association between elevated resistance to glucocorticoids (assessed using *in vitro* proliferation in the presence of exogenous glucocorticoids) and *in vitro* IL-1β production was noted. We elected to incorporate in our meta-analysis only studies that reported an *in vitro* measure of glucocorticoid resistance *and* plasma levels of cytokines in **Figure 5** and therefore, Maes et al. (72) was excluded from our analysis. Nonetheless, this study further

supports the idea that elevated glucocorticoid resistance in depression is associated with increased cytokine-based inflammation.

Initially we considered more stringency in our exclusion criteria, such as removal of all studies in which medical co-morbidities were present in patients and controls, but we later re-considered this decision when we uncovered a body of literature that demonstrated changes in glucocorticoid resistance in patients who were both depressed and afflicted with a chronic illnesses such as cancer (51, 52). We amended our approach to include study participants who may have suffered from medical conditions, so long as their inclusion was balanced by matching controls. We reasoned that this would increase the resolution of our work, and we believe that it did without materially biasing our results, as censoring of the four studies examining glucocorticoid resistance and cytokine production in depressed patients with a variety of medical illnesses [e.g., asthma (27), cancers (51, 52), and cardiac disease (45)] did not significantly change our conclusions (data not shown). Unfortunately, the heterogeneity of these four studies in terms of the medical illnesses displayed by study subjects and the lack of similar published works precluded a more detailed analysis of this facet of inflammation in depression. These findings highlight the complex interplay between affective illness and medical illness that was discussed earlier (3, 73–75).

In further efforts to include as many relevant studies as were available, we chose not to exclude the results from the two studies that examined older adults exclusively (45, 48). All other retrieved studies limited participants to those of adult ages yet pioneering studies of glucocorticoid resistance in the depressed elderly provided early evidence that standard measures of glucocorticoid resistance are valid in a population of advanced age, even in the context of elevated levels of dementia and chronic medical conditions (76, 77). Indeed, glucocorticoid resistance measured using the DST has been validated by meta-analysis to be able to distinguish severely depressed, psychotic individuals from those with more mild disease (78); elderly depressed patients are more likely to experience psychotic symptoms than their younger counterparts. We believe that inclusion of studies that examined both elderly patients and those with medical co-morbidities served to more faithfully model real-world facets of depression and allowed us to include potential results that may represent medical illnesses priming the HPA axis for increased dysfunction in the context of concurrent depression.

Our conclusions are limited by the small number of study subjects included in this review. This directly impacted the precision of effect size estimates, as did the variations in individual study design. Our latter analyses (**Figures 4–6**) were particularly at risk of such bias given the need to pool cytokine results. To rule out this possibility, we conducted the same analysis using varied combinations of cytokines from those reported in **Figures 4** and **5**, finding that these ancillary analyses did not differ significantly from the results reported (data not shown). Nevertheless, there is a need for large studies specifically designed to examine the association between glucocorticoid resistance and cytokine production in depression. These studies should include multiple objective measures of glucocorticoid resistance including cortisol levels, the DST, *GR* expression, and possibly, *in vitro* measures of GR function. A comprehensive characterization of glucocorticoid resistance could then be paired with an analysis of at least plasma levels of the major cytokines reviewed in this work (e.g., IL-6 and TNF-α). Inclusion of medication-free patients may be an important aspect to consider in the design of these studies also as levels of both cortisol and cytokines are modulated by antidepressant treatment (13, 79, 80). The work of Cattaneo et al. (56), which is included in this paper, represents an ideal design template from which to draw upon in future work. Here, medication-free depressed patients and controls were recruited, and expression of multiple cytokines as well as the *GR* was taken as part of a larger study examining immune predictors of antidepressant response. Addition of a measurement of plasma cortisol and a DST in each subject would have created a study tailor-made to examine the questions that we have asked in this review.

Two smaller relevant studies have been conducted that may help to fill this gap temporarily. Vedder et al. (81) elegantly assessed the response of the immune system in real-time before and after exposure to LPS. Using concurrent dexamethasone challenge, the authors demonstrated that in depressed patients who were glucocorticoid resistant, increased IL-6 was produced in response to LPS. This contrasted with IL-6 levels produced by patients with lower levels of glucocorticoid resistance and controls. Therefore, glucocorticoid resistance in depressed patients facilitated IL-6 release. Heiser et al. (79) took an *in vitro* approach to examining the same process. They report that immune cells from depressed patients that were resistant to the anti-proliferative effects of glucocorticoids *in vitro* produced more TNF-α in response to a mitogen than controls. Together with our work, these studies suggest that when glucocorticoid resistance is thoroughly quantified, support for its association with the increased production of pro-inflammatory cytokines can be found.

How depressed patients manifest high levels of inflammation in the face of elevated serum glucocorticoids is a psychiatric paradox. Our work demonstrates that we are still in the throes of disentangling this complex relationship. We believe that glucocorticoid resistance in depressed patients may lead the immune system to escape from the normally restraining function(s) of glucocorticoids. The results we present here provide some support for our hypothesis, but also highlight the need for further work. We are excited to be part of this ongoing search.

AUTHOR CONTRIBUTIONS

Conceived of study idea: AP, MH, PZ, CP. Conducted initial literature searches: MH, JR. Conducted literature searches, reviewed retrieved papers, and extracted study data: AP, MH. Performed meta-analysis: AP. Interpreted data: AP, CP. Wrote the first draft of the manuscript: AP. Revised further manuscript drafts in response to feedback from co-authors: AP. Provided project oversight and intellectual guidance: CP.

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REVIEW
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Multilevel Interactions of Stress and Circadian System: Implications for Traumatic Stress

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The dramatic fluctuations in energy demands by the rhythmic succession of night and day on our planet has prompted a geophysical evolutionary need for biological temporal organization across phylogeny. The intrinsic circadian timing system (CS) represents a highly conserved and sophisticated internal "clock," adjusted to the 24-h rotation period of the earth, enabling a nyctohemeral coordination of numerous physiologic processes, from gene expression to behavior. The human CS is tightly and bidirectionally interconnected to the stress system (SS). Both systems are fundamental for survival and regulate each other's activity in order to prepare the organism for the anticipated cyclic challenges. Thereby, the understanding of the temporal relationship between stressors and stress responses is critical for the comprehension of the molecular basis of physiology and pathogenesis of disease. A critical loss of the harmonious timed order at different organizational levels may affect the fundamental properties of neuroendocrine, immune, and autonomic systems, leading to a breakdown of biobehavioral adaptative mechanisms with increased stress sensitivity and vulnerability. In this review, following an overview of the functional components of the SS and CS, we present their multilevel interactions and discuss how traumatic stress can alter the interplay between the two systems. Circadian dysregulation after traumatic stress exposure may represent a core feature of trauma-related disorders mediating enduring neurobiological correlates of trauma through maladaptive stress regulation. Understanding the mechanisms susceptible to circadian dysregulation and their role in stress-related disorders could provide new insights into disease mechanisms, advancing psychochronobiological treatment possibilities and preventive strategies in stress-exposed populations.

Keywords: circadian system, circadian clocks, stress, trauma, HPA axis, autonomic nervous system, glucocorticoids, sleep

INTRODUCTION

Living organisms consist of highly complex biological systems with the ability to preserve a complex dynamic balance state with a constant oscillation around an ideal homeostatic condition (*nonequilibrium homeodynamic state*) (1, 2). To achieve this, organisms have developed a highly sophisticated and multifaceted biological system, the so-called stress system (SS), which serves self-regulation and adaptability of the organism to ongoing intrinsic or extrinsic, real or perceived (i.e., subject-dependent value attribution), altering challenges or stimuli, defined as stressors (3). When stressors surpass a manageable severity or temporal verge, the initiated stress response redirects energy depending on the present needs to restore homeostasis (4–8). Thus, stress is defined as the state of threatened homeodynamic balance of the organism (6, 9). Repeated, ephemeral, and motivating stress states lead to adaptive responses and are fairly beneficial, while inadequate, aversive, excessive, or prolonged stress may surpass the natural regulatory capacity and adjustive resources of the organism and majorly affect adaptive responses leading to *cacostasis (i.e., negatively altered homedynamic state, dyshomeostasis)*, and accumulated *cacostatic* load (6).

The understanding of the temporal relationship between stressors and physiological stress responses is crucial for the comprehension of the molecular basis of physiology and pathophysiology of disease. Biological processes always take place in an appropriate order, in order to synchronize required homeostatic mechanisms. As life on earth has evolved in the context of the earth's rotation around its own axis, there was a geophysical evolutionary need for temporal organization and adjustment of internal activity and physiological processes to the dramatic fluctuations in energy demands by the constant rhythmic succession of night and day. This need has generated a highly conserved and sophisticated internal molecular "clock," creating endogenous rhythmicity with a period adjusted to the 24-h rotation of our planet throughout phylogeny (10–12).

This intrinsic circadian (lat. *circa diem* – about a day) timing system (CS) creates an internal representation of the external *Zeitraum* (germ. time-space) and helps living organisms keep track of time from a centrally created circadian rhythm (13, 14). By orchestrating a dynamic milieu that oscillates with a 24-h rhythm, the CS coordinates physiological processes and rhythmic changes, from gene expression to behavior and prepares living organisms for the anticipated cyclic challenges, promoting homeostasis and environmental adaptation and creating an evolutionary advantage to optimize survival (15–18). In order to achieve this, the CS upregulates the SS before the organism's active phase and turns it down again for the resting and restorative phases.

The CS and the SS are both fundamental for survival and regulate each other's activity, through intimate reciprocal interactions with each other at multiple levels (19, 20). An intact communication between the CS and the SS is important for maintaining homeostasis and environmental adaptation (21–23). The SS is undoubtedly at the heart of circadian biology, mediating temporal signals and *vice versa* (24). Investigating the interactions between the two systems is essential to understand pathophysiological pathways mediating risk for disease, as dysregulation in either of these systems may lead to similar pathologic conditions (25).

In this review, following a general overview of the functional elements of the two systems, we present their multilevel interconnections, and discuss how excessive (i.e., traumatic) stress can affect the harmonic central and peripheral interplay between SS and CS.

THE HUMAN STRESS SYSTEM

The human SS consists of central and peripheral components. The central, critically interconnected components of the SS are mainly located in the hypothalamus and the brainstem, and include: (a) the parvocellular neurons of corticotropine-releasing-hormone (CRH), (b) the arginine-vasopressin (AVP) neurons of the hypothalamic paraventricular nuclei (PVN), (c) the CRH neurons of the paragigantocellular and parabranchial nuclei of the medulla and the locus caeruleus (LC), (d) the arcuate nucleus proopiomelanocortin-derived peptides alpha-melanocyte–stimulating hormone (MSH) and beta-endorphin, (e) other mostly noradrenergic (NE) cell groups in the medulla and pons (LC/NE system), and (f) the central nuclei of the autonomic nervous system (ANS) [cf. **Figure 1**]. These neuroanatomical loci communicate with each other, influencing their own activity, and interact with several other brain subsystems, such as the mesocortical/mesolimbic dopaminergic system, involved in reward and motivation and the amygdala central nuclei, generating fear and anger (6, 9).

The peripheral components of the SS include: (a) the hypothalamic-pituitary-adrenal (HPA) axis and (b) the ANS comprised of (i) the sympathetic nervous system (SNS) and sympatho-adrenomedullary (SAM) system and (ii) the parasympathetic nervous system (PNS). The main terminal peripheral effector molecules of the SS are the HPA axis-regulated glucocorticoids (GCs; i.e., cortisol in humans), and the SAM-regulated catecholamines (Cas; i.e., NE and epinephrine). HPA axis and ANS have largely complementary actions throughout the body and are increasingly studied together (26), as integrated and interrelated components of an internal neural regulation system. Findings suggest that the appropriate regulation of the HPA-axis depends in part on ANS, especially on vagal influences (27).

When stressors exceed a certain severity or temporal threshold, stressor-related information initiates a complex stress response to induce remarkably consistent acute, normally adaptive, and time-limited microphysiologic, mesophysiologic, and macrophysiologic compensatory responses throughout several effector tissues (4–8, 28). Together, these responses represent a well-orchestrated and fine-tuned answer to challenge in both the central nervous system (CNS) and the somatic periphery (29).

The Autonomic Nervous System

The ANS, although not under overt voluntary direction (*autonomous*), plays a crucial role in the preservation of a homeodynamic balance by providing a rapidly responding

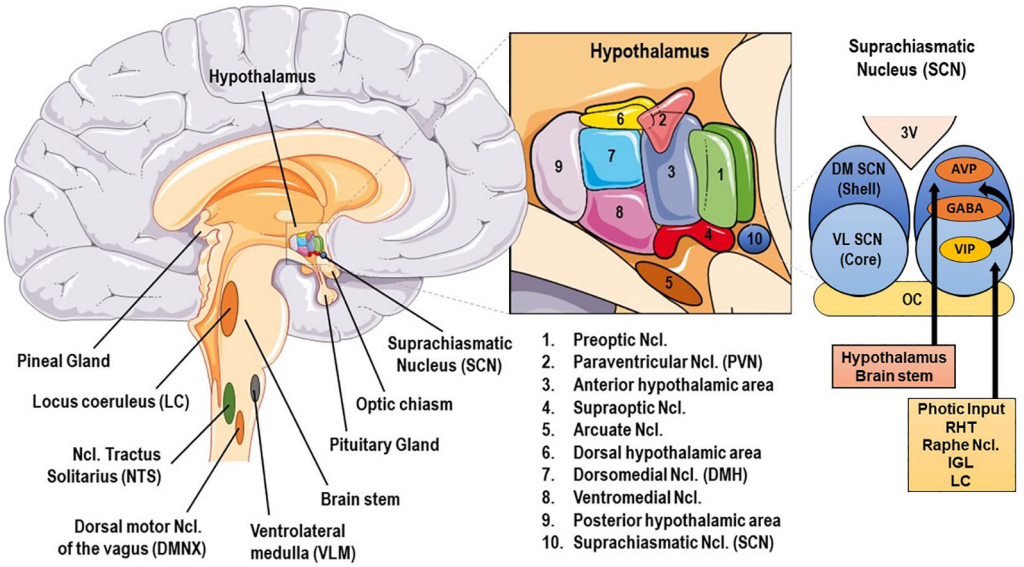

FIGURE 1 | Basic anatomy of stress and circadian system related brain structures. AVP, arginine vasopressin; GABA, γ-aminobutyric acid; DM SCN, dorsomedial suprachiasmatic nucleus; IGL, thalamic intergeniculate leaflet; LC, locus caeruleus; RHT, retinohypothalamic tract; VIP, vasoactive intestinal peptide; VL SCN, ventrolateral SCN.

control system for a plethora of physiological reactions to physical, emotional, and cognitive challenges (30, 31). It is especially the precise regulation of organ and tissue functions through fine-tuning of the ANS limbs that is crucial for optimal stress reactivity, adaptive responses, and health.

The exact ANS activity is fine-tuned through central and peripheral autonomic reflexes and feedback mechanisms (32). The central autonomic modulation does not simply rely on a monolithic network of brain regions, but is instead regulated by the central autonomic network (CAN), an internal central autonomic regulation system featuring certain task and division specificity (33). The CAN is additionally characterized by bilateral interconnections, parallel organization, state-dependent activity, and neurochemical complexity (30, 31, 34, 35). It includes the insular cortex, central nucleus of the amygdala, hypothalamus, periaqueductal gray matter, parabrachial complex, nucleus of the solitary tract (NTS), and ventrolateral medulla (VLM) (36, 37) [cf. **Figure 2**]. The insular cortex and amygdala mediate high-order autonomic control associated with cognitive perception and emotional responses through hypothalamic-brainstem pathways (30). NTS, PVN, and VLM contain a network of respiratory, cardiovagal, and vasomotor neurons, receiving afferent vagal sensory input from thoracic and abdominal viscera and other cranial nerves. These structures accordingly modulate the activity of preganglionic autonomic neurons. CAN dysregulation can be critically involved in stress-related disorders, as it may affect downstream autonomic centers, thereby altering peripheral ANS activity and cardiac function. CAN dysregulation (35, 38, 39) may affect downstream autonomic core centers, thereby altering peripheral ANS activity (39–41).

Since the early 20th century, pragmatic and anatomic reasons has led to a common division of the ANS into two, or sometimes three peripheral tracts: the sympathetic, parasympathetic and, the largest one, the enteric autonomic division, although they practically mirror one larger control system (42, 43). Especially the separation into SNS and PNS has led to enormous misconceptions, the most serious being the view that the two divisions are somehow in opposition to each other. On the contrary, SNS and the PNS are rather in a dynamic *interdependent* state and act on different time scales but in concert and through numerous and multilevel, bidirectional interactions to control the abovementioned autonomic functions (44, 45), while autonomic dysregulation translates into decreased dynamic adaptability, increased morbidity and mortality (27, 30, 46, 47). In general, since both systems are tonically active, the PNS can both assist and antagonize SNS functions by withdrawing or increasing its activity (frequency of neuronal discharge), respectively. This ANS characteristic is of major importance and improves its ability to more precisely regulate an effector's function.

The Sympathetic Nervous System

The SNS originates in brainstem nuclei and gives rise to preganglionic cholinergic (ACh) efferent fibers mostly projecting to postganglionic sympathetic ganglia. The long postganglionic neurons terminate outwards on effector tissues, mostly releasing NE. Alternatively, preganglionic neurons may also directly synapse with the modified postganglionic chromaffin cells of the adrenal medulla. A sympathetic activation, thus, principally releases NE (locally and to a lesser extent systematically from the adrenal medulla) or adrenaline (systematically from the

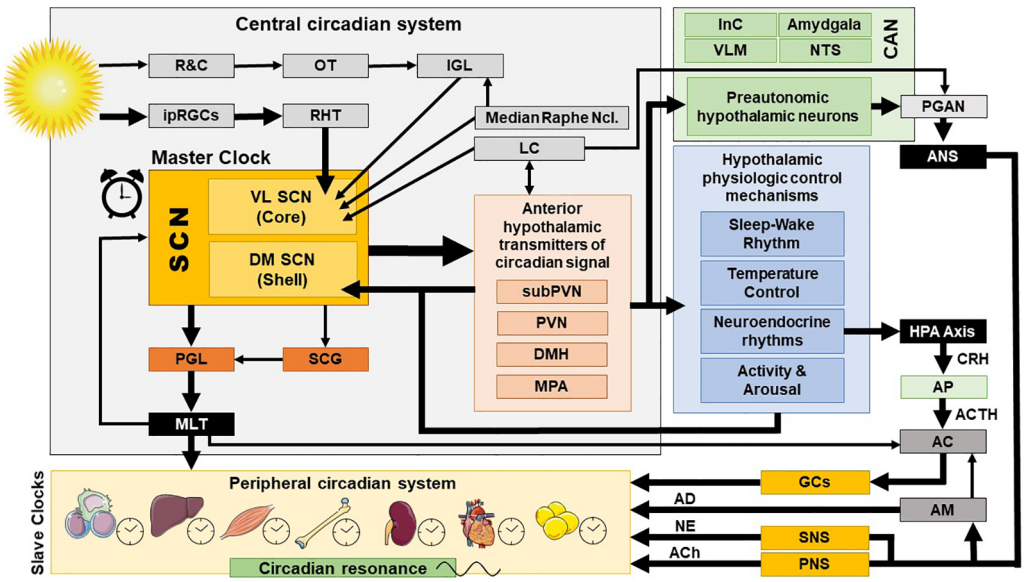

FIGURE 2 | Central and peripheral circadian system and their interconnections. AC, adrenal cortex; ACh, acetylcholine; ACTH, adrenocorticotropic hormone; AD, adrenalin; AM, adrenal medulla; ANS, autonomic nervous system; AP, anterior pituitary; CAN, central autonomic network; CRH, corticotropin releasing hormone; DM SCN, dorsomedial SCN; DMH, dorsomedial hypothalamus; GCs, glucocorticoids; HPA axis, hypothalamic-pituitary-adrenal axis; InC, insular cortex; IGL, thalamic intergeniculate leaflet; ipRGC, intrinsically photosensitive retinal ganglion cells; LC, locus caeruleus; MLT, melatonin; MPA, medial preoptic area; NE, norepinephrine; NTS, nucleus of the solitary tract; OT, optic tract; PNS, parasympathetic nervous system; PGAN, preganglionic autonomic neurons; PGL, pineal gland; PVN, paraventricular nucleus; R&C, rodes and cones; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; SCG, superior cervical ganlia; SNS, sympathetic nervous system; subPVN, subparaventricular area; VL SCN, ventrolateral SCN; VLM, ventrolateral medulla.

adrenal medulla) together with other neuropeptides in the body (48). Sympathetic activation generally predominates during emergency (*fight-or-flight*) situations and during exercise, preparing the body for strenuous physical activity.

The Parasympathetic Nervous System

Whereas SNS activity depends on two peripheral branches (neural and adrenal), parasympathetic activity is displayed only by nerves. The preganglionic neurons of the PNS arise from numerous brainstem nuclei and from the spinal sacral region (S2–S4). The preganglionic ACh-axons are quite long and synapse with short postganglionic neurons within terminal ganglia close to or embedded to effector tissues. Accordingly, PNS actions are mostly more discrete and localized compared to the SNS, where a more diffuse and global discharge is probable. The preganglionic neurons that arise from the brainstem exit the CNS through the cranial nerves [N. occulomotorius (III); N. facialis (VII); N. glossopharyngeus (IX); N. vagus (X)]. The vagus nerve innervates the thoracic and abdominal viscera and has a major physiological significance, as approximately ¾ of all parasympathetic fibers originate from the vagus nerve (49). The PNS stress response is mainly activated by the nucleus ambiguus and the dorsal motor nucleus of the vagus nerve, possibly after NTS stimulation. The PNS generally predominates during resting conditions towards conserving and storing energy or regulating basic body functions (e.g., digestion, defecation, urination). Through its tonic properties, the PNS is vital especially under resting conditions, and is, therefore, particularly implicated in the development of cardiovascular diseases and other comorbidities (27, 50).

The Hypothalamus-Pituitary-Adrenal Axis

The HPA axis consists of the PVN, the pituitary corticotrophs and the *zona fasciculata* of the adrenal cortex, which, respectively, employ corticotropin-releasing hormone (CRH)/arginine vasopressin (AVP), adrenocorticotropic hormone (ACTH), and glucocorticoids (GCs, i.e., cortisol in humans) as their signalling effector molecules [cf. **Figure 2**]. CRH and AVP are released from the PVN into the hypophyseal system in response to stimulatory signals from higher regulatory centers (e.g., PFC) and reach the pituitary gland to stimulate the secretion of ACTH. ACTH reaches the cortex of the adrenal glands through release in the systemic circulation and stimulates both production and secretion of GCs. Systemically released GCs, in turn, besides their major actions, close a negative feedback loop by suppressing the activation of the PVN and the pituitary gland (6, 51).

Glucocorticoid Receptors and Signaling

GCs influence a myriad of physiologic functions and are essential for the activation, maintenance, and downregulation of the stress response. GCs mainly exert their pleiotropic effects through genomic, nongenomic, and mitochondrial actions of the intracellular cognate GC and mineralocorticoid receptors (GR, MR), which function as a ligand-activated transcription factors (4–9, 52–56). GR and MR are evolutionarily close, showing large

homologies at their DNA-binding domain and sharing many responsive genes. Upon ligand-binding, the receptors dissociate from the interacting proteins (i.e., shock proteins and immunophillins), translocate to the nucleus, form homo- or hetero-dimmers and bind to specific DNA response elements located in the regulatory regions of thousands responsive genes, leading to their transactivation or transrepression (8, 52, 54–57). GR and MR have complementary actions with respect to HPA axis activity and reactivity (58). Altered GC-signaling, through dysregulations at different levels of the HPA axis, may greatly negatively affect the organisms' physiology and could influence life expectancy, as seen in many complex behavioral and somatic disorders (e.g., depression, posttraumatic stress disorder, sleep disorders, chronic pain and fatigue syndromes, obesity, diabetes Type II and the metabolic syndrome, essential hypertension, atherosclerosis, osteoporosis, autoimmune inflammatory, and allergic disorders) (55, 59).

In humans, the glucocorticoid receptor (hGR) is encoded by the *NR3C1* gene, which is located in the long arm of chromosome 5 and consists of 10 exons. The alternative usage of exon 9α or 9β gives rise to the two main receptor isoforms, the classic hGRα and the hGRβ (8, 52, 54–57). Ubiquitarilly expressed in every tissue except the suprachiasmatic nucleus (SCN) of the hypothalamus, the hGRα is primarily localized in the cytoplasm of glucocorticoid target cells (57, 60). hGRβ, exclusively localized in the nucleus of certain cells (e.g., endothelial cells), acts mainly as negative regulator of hGRα transcriptional activity (61, 62). A growing body of evidence suggests that hGRβ has its own, hGRα-independent transcriptional activity and plays an important role in insulin signalling, inflammation, and carcinogenesis (63). The MR is encoded by the *NR3C2* gene, is located on chromosome 4 and also consisting of 10 exons (64). MR is peripherally expressed in several tissues (e.g., adipose tissue, kidney, endothelium, macrophages) and exerts vital regulatory functions through its main endogenous MR ligand as part of the renin-angiotensin-aldosterone system, among others, in cell growth, renal and cardiovascular function, metabolism and immunity.

Of particular importance are the GR and MR effects in the CNS. While GR are expressed throughout the brain, MR are abundantly expressed in limbic brain structures involved in emotional processing, arousal and memory (i.e., hippocampus, amygdala, prefrontal cortex) thus exerting a basal inhibitory tone on GC secretion (65, 66). Interestingly, the MRs show a tenfold higher affinity to cortisol than GRs and are largely already occupied under basal cortisol levels, while GRs become gradually occupied through cortisol peak levels (e.g., circadian peak, acute stress) (58, 67), resulting in a regulative, MR-associated threshold for HPA axis activation and stress sensitivity (68). Thus, depending on receptor type, cell topology, tissue-specific expression, their specific ligands (e.g., aldosterone) or relevant enzymes (e.g., cortisol-inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2, 11βHSD2), HPA axis activation differentially regulates the expression of various target genes with different transcriptional potencies in response to cortisol.

In addition, GC may also signal through protein-protein interactions between receptors and other important transcription factors, including the nuclear factor-κB (NF-κB), the activator protein-1 (AP-1), and the signal transducers and activators of transcription (STATs). However, perhaps even more importantly, GC exert also rapid, nongenomic actions, mediated by membrane-bound MRs and GRs that trigger the activation of kinase signal transduction pathways (8, 52, 54–57, 69). Membrane-bound MRs and GRs show lower GC affinity than intracellular receptors and are increasingly occupied only through higher cortisol concentrations, thus mainly playing a crucial role in translation of rapid GC pulses in the initial phase of HPA axis activation (70–72).

THE HUMAN CIRCADIAN SYSTEM

Circadian molecular oscillations are independently generated in virtually every cell of living organisms, thus influencing molecular biological processes over the course of the day. However, it is the orchestration of these innumerable, diverging and tissue-specific peripheral oscillations into a main rhythmic symphony that is of vital importance for the promotion of homeostasis in higher organisms. The CS represents an extensive network of time-keeping mechanisms that creates and maintains this cellular and systemic rhythmicity, through temporal organization and coordination of many physiological and transcriptional oscillating processes throughout several structural levels in the organism (17, 18). In order to stay adjusted to the geophysical time, the CS receives continuously input by behavioral, hormonal, and environmental signals, a process called entrainment.

The mammalian CS is organized in a hierarchical manner with a central, pacemaking, and light-sensitive "master clock" in the CNS and a peripheral, subordinated multioscillator component ("slave clocks"), showing both top-down and bottom-up organization based on positive and negative endocrine, autonomic, and transcriptional regulatory feedback loops (15, 73–75). The CS has three main functions as (a) pacemaker through intrinsic and self-sustainable rhythm generation, (b) internal *Zeitgeber* (*germ*. time-giver) with a distinct rhythm output for peripheral synchronization, and (c) *Zeitnehmer* (*germ*. time-taker) continuously receiving time-shifting signals from external/secondary *Zeitgebers* (e.g, nutrition, light, sleep, social activity) for proper time entrainment of the intrinsic period to the environmental cycle (76).

The Central and Peripheral Circadian System

The central mammalian CS includes specialized signal transduction mechanisms in the retina, the retinohypothalamic tract (RHT), the suprachiasmatic nucleus (SCN), the superior cervical ganglia, the pineal gland (PGL), the thalamic intergeniculate leaflet (IGL), and the raphe nuclei (18, 77, 78) (cf. **Figures 1** and **2**). The SCN is a bilateral paired structure with

high cell density, consisting of 50,000 neurons (in humans) displaying a synchronised rhythmic metabolic and electrical activity, and is located in the anterior hypothalamus directly over of the optic chiasm, next to the third ventricle. The SCN is the integrative "master clock" of the organism, by integrating its distinct primary pacemaker activity through intrinsic neural firing and all received environmental *Zeitgeber* cues to a main circadian rhythm (17, 18, 79–81). The most important *Zeitgeber* is light. The SCN receives photic input (photoentrainment) from the rod/cone photoreceptors and particularly from other nonimage-forming photosensitive cells in the retina, the intrinsically photosensitive retinal ganglion cells (ipRGCs) (77). These melanopsin-containing cells have been shown to be sensitive to light wavelengths (460–480 nm, i.e. blue light) different from the classical visual system (i.e., rod and cone cells) and they react slowly and tonically to luminance changes (77, 82–87). The photic input transmitted from the ipRGC through the retinohypothalamic tract to the SCN (88) and from there to the upper part of the thoracic spinal cord, the superior cervical ganglia and the PGL gland (89). The NPY-containing pathway from the IGL and the serotonergic pathway from the median raphe represent the two other main afferent projections to the SCN (78). Taken together, anatomical routes directly involved with the SCN are numerous, with up to 15 efferent and 35 afferent projections (78).

The peripheral, subordinated multioscillator component of the CS ("slave clocks") show a similar, tissue-specific, self-sustained, and cell-autonomous rhythm generation machinery, regulating several functions of their residing tissues, with one essential difference to the central CS: These peripheral "slave clocks" do not exchange phase information and must therefore kept synchronized by the main integrative SCN rhythm *via* different pathways (16), which leads to a 4-h optimal phase synchronization delay of peripheral with respect to the central CS rhythm (90). This synchrony gets mostly lost without an input from the SCN (91), although other *Zeitgebers,* such as nutrient, temperature, and social cues, can also entrain peripheral clocks (92).

The Molecular Clockwork

In the past decades, mounting evidence has evolved our understanding from the first discovered clock gene (Period or PER) conserved from fruit flies to humans (93) to a complex molecular clockwork generated at the cellular level by molecular oscillators in all nucleus-containing cells of an organism (15, 74, 94). The intrinsic circadian rhythmicity of the biological clock is based on a core set of clock genes intertwined with an autoregulatory, delayed, interlocking transcriptional/translational feedback (TTFL) loop machinery, coupled to several auxiliary mechanisms and leading to mutual transcriptional activation and repression, ultimately maintaining an approximately 24-h oscillation, thus, reinforcing robustness and stability of the clock (14, 15, 74, 94–97).

Central among the core TTFL are the transcriptional activator "circadian locomotor output cycle kaput" (CLOCK), its heterodimer partner "brain-muscle-ARNT-like protein 1" (BMAL1), and the essential negative regulating circadian genes "Period 1, 2, and 3" (PER1-3) and "Cryptochrome 1 and 2" (CRY1/2) (98). The activated CLOCK/BMAL1 heterodimer binds to the enhancer box (E-box) response elements located in the promoter region and stimulates the transcription of PER1-3 and CRY1/2 at circadian dawn (circadian time 0, CT0). PER1-3 and CRY1/2 mRNA gets translated into proteins, which accumulate by the end of the circadian day (CT12). Over the course of the circadian night (CT12–CT0), inhibitory complexes of PER1-3 and CRY1/2 with the caseine kinase 1ε and δ, are phosphorylated and translocate from the cytoplasm into the nucleus and repress the transcriptional activity of the CLOCK/BMAL1 in the SCN, shutting down PER1-3/CRY1/2 transcription (99). After degradation of nuclear PER1-3/CRY1/2 complexes the next morning (CT0), the inhibition on CLOCK/BMAL1 transcriptional activity is released and thereby a new cycle starts over after approximately 24 h (79) [cf. **Figure 3**]. During the circadian day, PER1-3 and CRY1/2 transcription is high in the SCN, leading also to high SCN electrical activity. Besides this core negative feedback loop, there are also auxiliary feedback loops that stabilize the transcriptional activity of the core regulatory loop (94, 100–102). CLOCK/BMAL1 upregulates, for example, the expression of other clock-related proteins, such as the reverse viral erythroblastosis oncogene product α and β (REV-ERBα/β) and the retinoic acid receptor-related orphan receptor α (RORα), which, in turn, regulate BMAL1 expression. Genetic polymorphisms in these clock genes are responsible for a great distribution of entrained phases (chronotypes) between individuals, ranging from "larks" to "owls," with most individuals falling between these extremes (103).

The transcription factors of both principal and auxiliary TTFLs can modulate the expression levels of many clock-responsive genes in various tissues, influencing a broad spectrum of physiologic functions, such as hormonal fluctuations, sleep/wakefulness, feeding, immune activity, thermoregulation, energy household, and glucose metabolism (14). These regulatory loops, receive adjustive input from related influencing systems. Besides the strongest circadian entrainment by light, other biological cues, such as nutrition and temperature, can also influence the activity of the clock system. For example, peripheral clocks can be influenced by food-related signals through adenosine monophosphate-activated protein kinase (AMPK), a tissue sensor and master regulator of energy balance, which phosphorylates Per1-3 and Cry1/2 leading to their degradation (104, 105). Similarly, temperature decrease can represent a strong circadian cue, as the cold-inducible RNA-binding protein CRBP accumulates under lower body temperature in peripheral clocks (but not in the SCN) and influences circadian gene expression (106).

Circadian System Interconnections and Effector Pathways

The superior robustness and resilience of the distinct intrinsic activity rhythm of the SCN is mainly preserved by the synchronization of SCN neurons through intercellular coupling to its neighbour cells in an action-potential-dependent manner (107). There are different kinds of SCN neurons containing

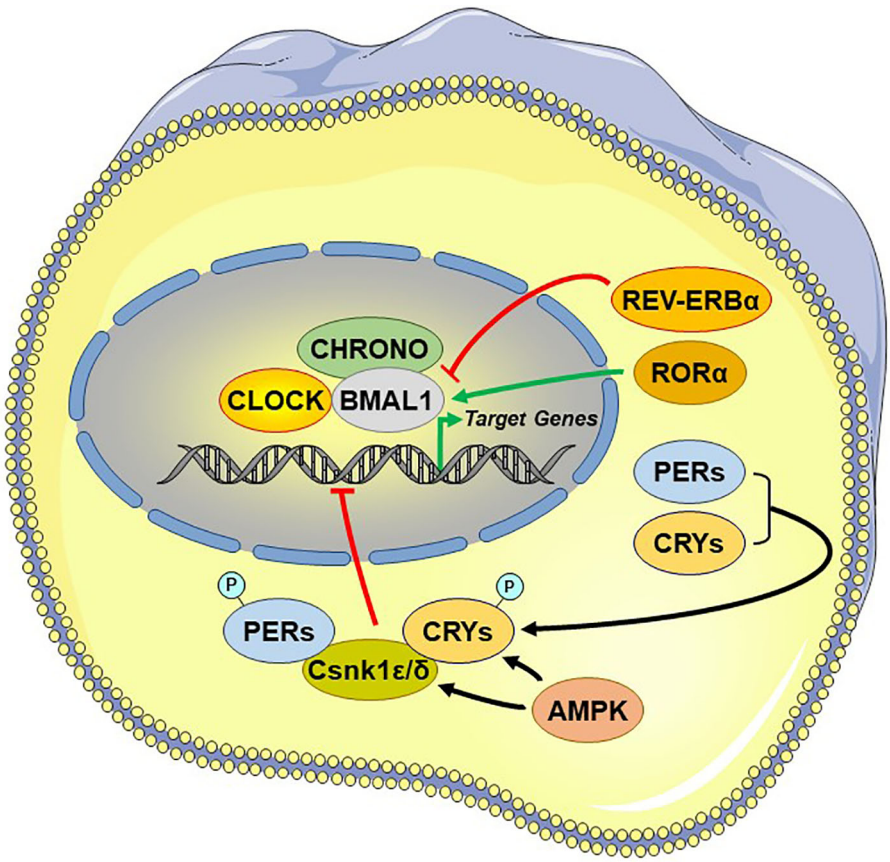

FIGURE 3 | Principal and auxiliary transcriptional/translational feedback loops of the circadian system. AMPK, adenosine monophosphate (AMP)-activated protein kinase; BMAL1, brain-muscle-arnt-like protein 1; CHRONO, ChIP-derived repressor of network oscillator; CLOCK, circadian locomotor output cycle kaput; CRYs: cryptochromes; Csnk1ε/δ, casein kinase 1ε/δ; P, phosphate residue on the phosphorylated molecules; PERs, periods; RORα, retinoic acid receptor-related orphan nuclear receptor α. REV-ERBα, reverse viral erythroblastosis oncogene product alpha.

different neuropeptides, such as arginin-vasopressin (AVP), vasoactive intestinal peptide (VIP), γ-amino-butyric-acid (GABA), glutamate, gastrin-releasing peptide, and somatostatin. This large variety of neuropeptides within the SCN ensures a rich diversity in signalling properties to effector targets (108). According to its neurocircuit topology, the SCN can be functionally divided into two subregions. The dorsomedial shell region primarily produces AVP and gets mainly innervated by the hypothalamus, while the ventrolateral core region primarily produces VIP and receives photic input [cf. **Figure 1**]. SCN output projections target many different brain regions and modulate the activity of downstream neurohumoral pathways in a rhythmic manner, herewith influencing a plethora of physiological processes (14, 16, 109). The most important effector targets of the SCN include: (i) hypothalamic centers associated with activity, temperature, and sleep regulation, such as the subparaventricular area (subPVN) and the dorsomedial nucleus of the hypothalamus (DMH) (110), (ii) preautonomic hypothalamic neurons, affecting vagal and sympathetic autonomic centers in brain stem and spinal cord and, thus, exerting circadian control throughout the body *via* ANS activity (80), and (iii) neuroendocrine hypothalamic centers responsible for hormone secretion (e.g., CRH synthesizing PVN parvocellular neurons) [cf. **Figures 2** and **5**]. The PVN is a significant integrating center for energy homeostasis and distribution center of circadian rhythmicity to the body, as its parvocellular neurons project to the median eminence to control the release of ACTH and thyroid-stimulating hormone (TSH) in the anterior pituitary (i.e., hypothalamic-pituitary-adrenal axis, HPA axis; hypothalamic-pituitary-thyroid axis, HPT axis), and also innervates the sympathetic limb of the ANS (22).

In addition, the central CS, exerts its synchronizing effects also through humoral (i.e., endocrine/paracrine) signals. The main effector of the central CS and essential synchronizing hormone is pineal melatonin (MLT) (111–114), whose secretion is strictly modulated by the SCN and sympathetic fibers originating from the superior cervical ganglia (112, 113, 115–117). Reversely, MLT is a direct modulator of the SCN neuron electrical activity (118, 119), as SCN expresses a high number of MLT receptors (MT) (120), while it also interacts with "clock" gene TTFLs in the SCN, and so modulates circadian rhythms and adjustment to environmental photoperiod changes

(121). MLT modulates central and peripheral oscillators and related secondary molecular pathways mainly by cell-specific control through G-protein-coupled MLT membrane receptors MT_1 and MT_2 (118) and GABAergic mechanisms (119, 122, 123) [cf. **Figure 4**]. MT is broadly distributed in the body and are vital for immunomodulation, endocrine, reproductive and cardiovascular regulation, cancerogenesis, and aging. Additionally, MLT interacts with cytoplasmic factors (i.e. quinone-reductase-II/MT_3 receptors, calmodulin) and nuclear receptors (i.e. retinoid acid receptor related orphan and Z receptors, ROR, RZR), while numerous other actions of MLT are receptor independent (e.g., radical scavenging) (114, 124–128). MLT concentration reaches high levels at night (plasma peak between 0200 h and 0400 h), overlapping with decreases in core body temperature, alertness, and performance (111, 113). The sharp elevation of nocturnal cerebrospinal fluid (CSF) MLT exerts substantial protective effects and is responsible for nocturnal tissue recovery after the daily free radical brain damage due to high oxygen utilization (129). These multifaceted chronobiotic regulatory actions have led to the recognition of MLT as one of the most pleiotropic biological signals in photoperiodic species (114, 130). On the other hand, it is important to note that the majority of laboratory mouse strains do not produce melatonin and thus challenge the importance of MLT in related animal findings (131).

Finally, sleep acts restorative in concert with the CS, but also independently, towards optimizing the internal temporal order (132). Sleep propensity and sleep stage timing, regulated through the subPVN and DMH, are bidirectionally associated with circadian gene expression in the SCN (133), but also strongly modulated by MLT levels (119, 134–138).

INTERACTIONS BETWEEN THE HUMAN CIRCADIAN AND STRESS SYSTEM

The human CS and SS are closely and bidirectionally interconnected at multiple central and peripheral functional

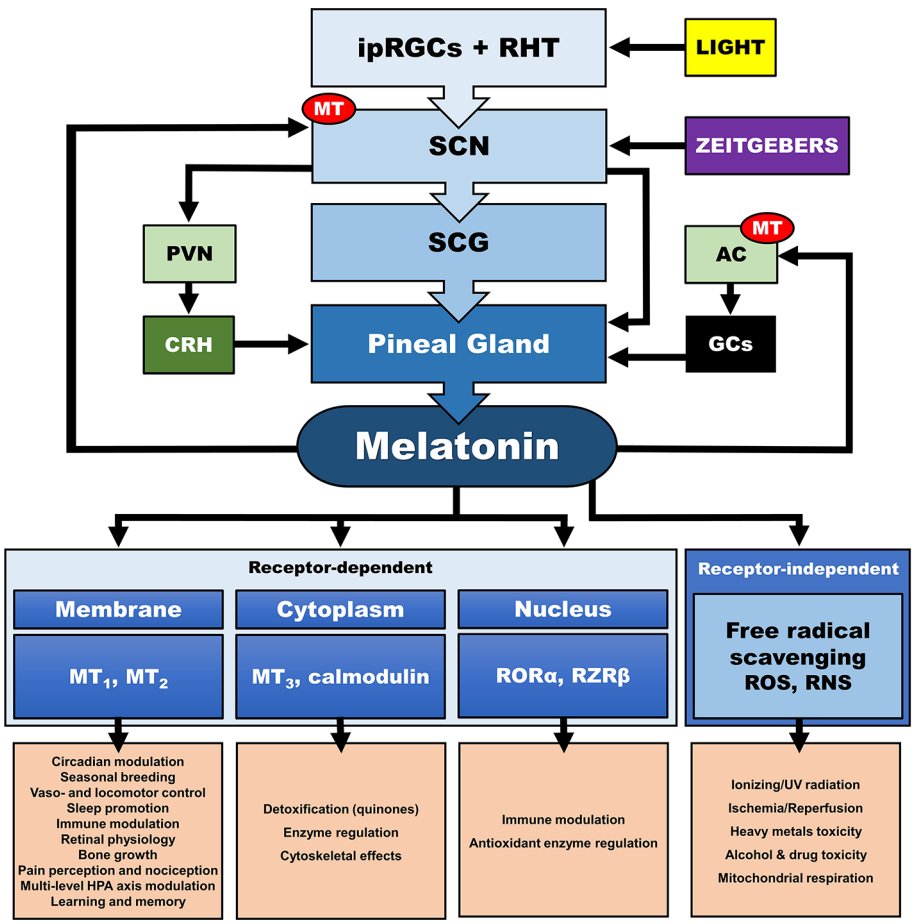

FIGURE 4 | Multilevel interactions between the circadian system and the hypothalamic-pituitary-adrenal (HPA) axis. AC, adrenal cortex; CRH, corticotropin releasing hormone; GCs, glucocorticoids; ipRGC, intrinsically photosensitive retinal ganglion cells; MT, melatonin receptor; PVN, paraventricular nucleus; RHT, retinohypothalamic tract; RNS, reactive nitrogen species; RORα, retinoic acid receptor-related orphan receptor α; ROS, radical oxygen species; RZRβ, retinoid acid receptor related Z receptor β; SCN, suprachiasmatic nucleus; SCG, superior cervical ganlia.

levels (19, 22, 23, 139–148). The circadian properties of the HPA axis are so distinct, that, along with MLT, GCs have been established as a robust measure of CS output activity. Additionally, MLT and GCs can also feedback at various levels and influence the main circadian rhythm themselves. Interestingly, the phase angle between CORT and MLT onset, the two major hormonal output signals of the CS and the HPA axis, has been identified as a potential useful biomarker in human stress-related research (149).

Influence of the CS on SS Activity and Reactivity

The HPA axis shows distinct circadian activity at rest with a robust diurnal oscillation of circulating GCs (i.e., cortisol, CORT) concentrations, rapidly rising in the middle of the biological night and peaking in the early morning, reaching their nadir before the habitual inactive phase onset (19, 141, 142, 150). SCN ablation completely abolishes the GC circadian rhythm, suggesting that HPA axis activity is driven by the central CS (151). In addition, the CS has a major influence on the ANS. Major human cardiovascular markers, such as heart rate, blood pressure, baroreflex, heart rate variability (vagal measure), plasma epinephrine, and norepinephrine levels (sympathetic measure) and their response to stressors exhibit robust circadian variations with a distinct peak of sympathetic activity and nadir of parasympathetic activity in the morning hours (152–157). By doing so, the HPA axis and SNS activity are believed to prepare the organism for the higher energetic demand associated with typical external and internal stressors of the waking phase (24).

Neurohumoral Interactions

The CS orchestrates the circadian activity and reactivity of the HPA axis through both hormonal and neuronal pathways. There are three main pathways of CS influence on the HPA axis: (i) direct SCN influence on HPA axis at the hypothalamic level, (ii) SCN innervation of the adrenal glands through indirect, multisynaptic autonomic innervation, and (iii) peripheral rhythms of local adrenal clocks, all three involved in the steroidogenic pathway and the ACTH-dependent transduction cascade in the zona glomerulosa and zona fasciculata of the adrenal gland (158) [cf. **Figures 2** and **5**]. The first pathway includes direct and indirect (through subPVN and DMH) neuronal projections of the SCN to CRH/AVP containing neurons of the medial parvocellular PVN modulating the circadian secretion of CRH and AVP (108, 146, 159, 160). Through the second pathway, the SCN transmits photic information *via* multisynaptic autonomic innervation (i.e., preganglionic intermediolateral projections to the spinal cord and splanchnic nerve innervation) to the adrenal medulla and

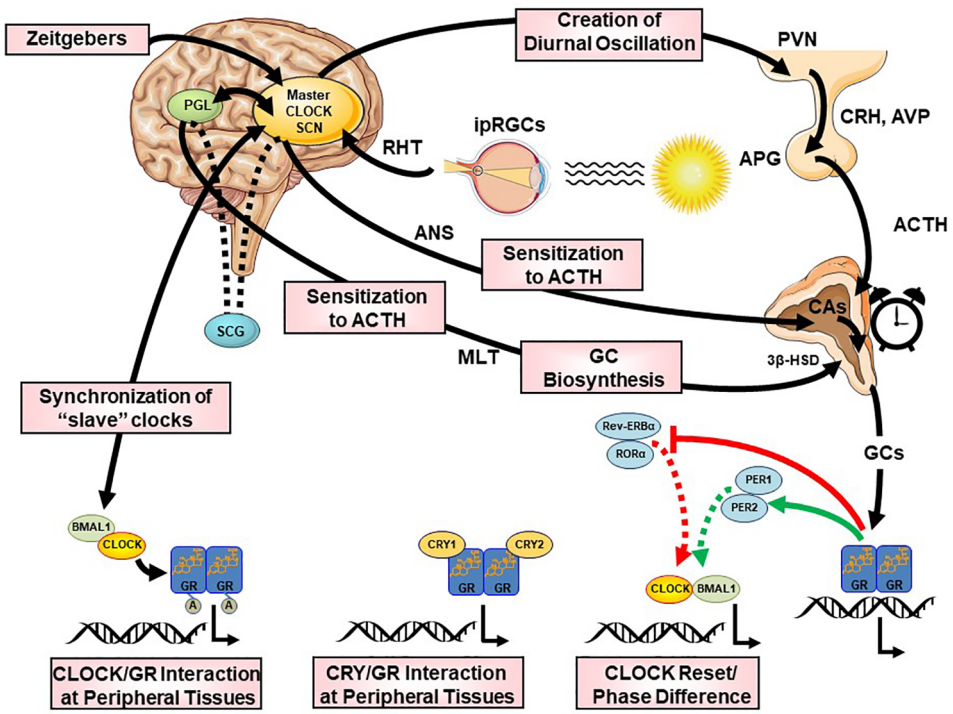

FIGURE 5 | Multilevel interactions between the circadian system and the hypothalamic-pituitary-adrenal (HPA) axis. ACTH, adrenocorticotropic hormone; APG, anterior pituitary gland; AVP, arginine vasopressin; BMAL1, brain-muscle-arnt-like protein 1; CA, catecholamines; CLOCK, circadian locomotor output cycle kaput; CRH, corticotropin releasing hormone; CRYs, cryptochromes; HSD, hydroxysteroid dehydrogenase; ipRGC, intrinsically photosensitive retinal ganglion cells; GCs, glucocorticoids; GR, glucocorticoid receptor; PERs, periods; PVN, paraventricular nucleus; REV-ERBα, reverse viral erythroblastosis oncogene product alpha; RHT, retinohypothalamic tract; RORα, retinoic acid receptor-related orphan nuclear receptor alpha SCN, suprachiasmatic nucleus.

from there through catecholamines to the cortex (161), thus both modulating the diurnal ACTH sensitivity of the adrenal cortex and stimulating the GC circadian release in light exposure conditions through an HPA axis-independent manner of direct interaction with the own peripheral rhythm of the adrenal gland (i.e., PER1 and StAR gene expression) (7, 140, 162–169). Interestingly, SCN neurons display connections to SNS and PNS, indicating that the SCN is not only essential for the physiologic autonomic diurnal fluctuations seen in humans (153, 155, 157), but also involved in both activation and deactivation of neuronal innervation of the adrenal in a circadian circle (80). The intrinsic circadian rhythm of adrenal glands in metabolic activity and GC release even in culture has been shown very early in literature (170), while clock genes expression was repeatedly reported in the following years (140, 164, 165, 171, 172). However, additional adrenal-intrinsic mechanisms depending on systemic cues, such as food-entrainable oscillators of the gland, could influence the diurnal rhythms of GC secretion (173, 174). Another very important mechanism for shaping the GC circadian rhythm is their own systemic levels, exerting a negative feedback regulation of ACTH release (175). The sensitivity of this feedback mechanism is highest during the trough point of the circadian glucocorticoid rhythm depending only MR at this time, while both MR and GR are involved at the GC peak-point lowest sensitivity (175). Finally, MLT, apart from its direct modulating effect on the SCN (176), has been also shown to directly influence GC production and release by the adrenal gland, as well as acetylation rhythms of GR, GR translocation to the nuclei and transcriptional activity (125, 172, 177, 178). MLT has been found to prevent adrenal response to ACTH (177, 179) and directly inhibit CORT production through MT1 adrenal receptor activation, possibly through their action on the Type II 3β-HSD (3β-Hydroxysteroid-dehydrogenase/$\Delta^{5\text{-}4}$ isomerase) enzyme activity, which catalyzes the biosynthesis of hormonal steroids through the oxidation and isomerization of Δ^5-3β-hydroxysteroid precursors to Δ^4-ketosteroids (180). Taken together, this illustrates the multilevel circadian "gating" control on the physiological GC secretion rhythm through SCN, HPA axis and ANS activity, GC and MLT levels, feeding and the robust intrinsic rhythm of the adrenal gland itself, involving clock gene expression in the metabolism and secretion of GCs (80, 140, 141).

In addition, MLT acts directly through MT_1/MT_2 on the electrical activity in SCN neurons (118, 119) and interacts with the "clock" gene (PER1/2, CRY1/2, CLOCK, BMAL1, etc.) proteasome TTFL in the SCN, thus being crucial for circadian entrainment in photoperiodic species (121).

Molecular Interactions

The neurohumoral interactions between CS and SS described above, have further molecular underpinnings at the cellular level, where the GR plays a fundamental role. For example, findings suggest that the CLOCK/BMAL1 heterodimer behaves as a reverse-phase negative regulator of hGRα in the periphery, antagonizing the physiologic actions of diurnally fluctuating GCs. Through a region enclosed in the C-terminal part of the CLOCK protein, CLOCK/BMAL1 physically interacts with the ligand-binding domain of hGRα and acetylates the hGRα at multiple lysine residues, thereby reducing GR's affinity to its cognate glucocorticoid response elements (GREs) and, thus, leading to decreased hGRα-induced transcriptional activity of glucocorticoid-responsive genes (144, 181–184). GR transactivational activity fluctuates in a circadian fashion and in reverse phase with CLOCK/BMAL1 mRNA expression (182) and leads to a higher hGRα acetylation and decreased tissue glucocorticoid sensitivity in the morning, mirroring the circadian pattern of serum CORT concentrations (183). In addition, a CLOCK-mediated posttranslational modification of hGRα is involved with the nuclear localization signal 1 (NL1), altering the cytoplasm-to-nucleus translocation of the receptor following ligand-induced activation, and indicates that the hGRα acetylation by CLOCK is linked to several molecular mechanisms (182). Moreover, Lamia and collaborators demonstrated that CRY1/2 interacted with the carboxyterminal domain of hGRα, thereby reducing the DNA-binding of the receptor and its transcriptional activity (185). Interestingly, the effect of a specific clock gene deletion on circulating GCs seems to depend on the specific TTFL missing member, suggesting that alteration of the positive or negative limb of the core clock feedback loop may have opposing effects on stress regulation. Accordingly, BMAL1 (TTFL positive limb gene) deletion leads to low adrenal ACTH sensitivity throughout the circadian circle, supporting constant low GC levels and insensitivity to acute stress (186). Genetic deletion of CRY1/2 (TTFL negative limb genes) leads to nonoscillating and elevated GC levels due to impaired feedback inhibition (185, 187). In contradistinction, the PER1/CRY1 complex reduces the maximal GR transactivation but not the efficacy of the receptor (184). Furthermore, CHRONO (ChIP-derived repressor of network oscillator), which is encoded by a BMAL-target gene, interacted with BMAL1, CRY2 and DEC2 and recruited the histone deacetylase 1 (HDAC1) to the transcriptional machinery, ultimately repressing the principal transcriptional loop (188). CHRONO is also able to acetylate the hinge region lysine cluster of GR, reducing its DNA-binding and thus indicating that this protein might play a fundamental role in the interaction of the CS with the SS (182, 188, 189). More recent *in vitro* and *in vivo* studies also showed that REVERBa, in interaction with heat-shock-protein (HSP) 90, influences the stability and nuclear localization of GR in the liver and provides another link between the CS, metabolism and glucocorticoid actions (190, 191). In addition, transcriptional cofactors of nuclear receptors (e.g., PGC1a) has recently been also implicated in circadian clock function (192), while interacting with the GR (193). Similarly, HSP, forming a dynamic complex with the GR in the cytoplasm (i.e., before GC binding and nuclear translocation), also display a circadian regulation through systemic circadian temperature changes, thus contributing to clock entrainment in peripheral tissues (194, 195). Finally, FKBP5, a chaperone protein of particular interest involved in directing activated GRs to the nucleus and implicated in a number of stress related psychiatric

disorders, is also rhythmically expressed in most tissues (196), suggesting its involvement in circadian gating of GC signals.

Influence of the SS on the Central and Peripheral CS

Apart from the influence on many important biological processes, the rhythmic oscillations of the SS activity and especially the HPA axis and GC rhythmicity exert a vital synchronizing effect on the central and peripheral CS activity (19, 23, 92). GCs, through binding to the hGRα, can efficiently reset the activity of peripheral clocks (197–199), while they spare the SCN, which maintains its master intrinsic circadian rhythm, as it does not express GRs (158, 197). The attenuation of the peripheral clocks by the phase-shifting effects of the GCs is then normally restored by the influence of the SCN. However, the SS has to directly influence the SCN through an alternative pathway, as both stress exposure and exogenous GC application enhances AVP and VIP mRNA expression and release in the SCN (200, 201), while acute stress exposure also leads to an upregulation of Per1 and Per2 protein expression in the SCN (202). For example, CORT and CRH are suggested to directly modulate PGL activity and stimulate MLT synthesis, interfering in the daily adjustment of the light/dark cycle (179, 203–206). In addition, GCs play an important role in the adjustment of nutrition-related uncoupling between the central and peripheral CS, as their high secretion after feeding slows down the circadian uncoupling and restores proper phasing (173, 207). GCs are, thus, not just a downstreal hormonal output of central and peripheral clocks, but can also influence the CS itself and interact with other clock outputs toward a harmonious circadian regulation (141, 197), adding another interaction level between the stress and the circadian clock system. Alteration of the GC rhythm (e.g., through exogenous GC administration) can, thus, attenuate the central and peripheral circadian activity and vice versa (167, 208). Taken together, the SS through its effectors efficiently adjusts the circadian rhythm-linked output pathways of the body to properly respond to stressors, providing resistance to stress challenges in order to evade uncordinated circadian shifts (23).

Molecular Interactions

Diurnally circulating GCs vitally contribute to the development of the CS activity by adjusting the phase of peripheral oscillators (19, 148). GCs synchronizing effects mainly involve GR-related phase shifting of peripheral circadian expression of several clock-related genes (197, 209–216). All peripheral clocks express GR, which translocate into the nucleus after activation and modulate transcriptional activity of several clock genes (e.g., PER1/2) and transrepressing genes expressing transcription factors of the auxiliary TTFL (e.g., Rev-ERBα, RORα) through binding to functional GREs in their promoter region (217–222). PER1 contains GRs in its regulatory sequences, while GRs influence the expression of PER2 through binding to an intronic domain (218). GCs lead herewith to upregulation of these genes, causing a phase delay of peripheral clocks with respect to the SCN master clock (218). A genetically, functionally (e.g., adrenalectomy) or pharmacologically (i.e. externally administered corticosteroids) attenuated GC diurnal rhythm has been shown to be associated with abolished or shifted circadian clock gene (e.g., PER1/2) expression in several peripheral tissues (e.g., liver, preadipocytes, kidney, bronchial epithelial cells, pancreas, bone tissue, cornea, fibroblasts cardiac muscle tissue), despite the presence of an intact molecular oscillator (167, 197, 208, 209, 215, 216, 223–226). Even externally applied corticosteroids can entrain molecular oscillation in peripheral clocks (215, 227) and have been shown, for example, to speed up or slow down adaptation to a new light-dark schedule after jetlag-induced circadian desynchrony (198).

However, rhythmic GC signaling is also required for periodic clock gene expression in certain brain regions outside the SCN, suggesting an important role of the adrenal rhythm also for higher brain functions in key stress-system-related regions (228). Indeed, GR-mediated GC signaling is, for example, fundamental for the rhythmic expression of PER2 in the amygdala (213, 229), while adrenalectomy is shown to supress and extended GC exposure to increase PER gene expression in the PVN, bed nucleus of stria terminalis (BNST) and other limbic areas (219, 228, 230–232). GC-dependent circadian gene expression could even be indirectly involved in a GC feedback pathway to the SCN (233). For example, serotonergic projections of the raphe nucleus to the SCN involved in light entrainment (234) show a GC-dependent circadian transcription of tryptophan hydroxylase-2 (TH-2), an enzyme involved in serotonin synthesis (235).

Finally, the SAM/ANS constitutes another pathway in stress-induced peripheral circadian entrainment. Administration of adrenaline or noradrenaline has been shown to induce PER1/2 expression through the cAMP response element-binding protein (CREB) signalling pathway (236–238). Furthermore, GR-related GC effects and clock machinery also interact through a modulation of catecholamine biosynthesis and degradation, thus influencing time-of-day-dependent stress responses and further reinforcing the interaction between the CS and the SS (94, 239, 240). Catecholamine biosynthesis is both GC- and clock-regulated, as TH (i.e., the main synthesis pacemaker enzyme) is repressed by Rev-ERBα (241) and induced by the GR-activated the nuclear orphan receptor NURR1 (NR4A2) (242). Similarly, catecholamine degradation depends on the CLOCK/BMAL1-activated monoamine oxidase I (MAO-A) and the GR-regulated catechol-O-methyltransferase (COMT) (243).

Taken together, GC rhythms exert an accompanying circadian signal which consitutes an additional level of security to ensure proper circadian signalling input to the cell cycle oscillating machinery, while, on the other hand, peripheral clocks might gate this GR-specific input.

STRESS AND CIRCADIAN MISALIGNMENT

Chronodisruption and Sleep Dysregulation

The human CS enables the nyctohemeral organization and coordination of many temporal physiologic processes promoting homeostasis and environmental adaptation (18). A

misalignment of the human circadian rhythm is associated with a critical loss of this harmonious biological timed order at different organizational levels, which is defined as *chronodisruption* (244–246). Chronodisruption-related cacostatic load with short- and long-term pathophysiologic and epigenetic consequences (245–247) can lead to a wide range of biological consequences in the organism (246, 248–255). Chronodisruption may gradually change the fundamental properties of brain systems regulating neuroendocrine, immune, and autonomic function and denotes a breakdown of appropriate biobehavioral adaptations to challenges with increased stress sensitivity and vulnerability to stress-related disorders (20, 256, 257).

In human research, chronodisruption has been tightly associated with sleep deprivation/dysregulation (SD) or phase shifting (i.e., jet-lag, swift-workers) (81, 132, 258). Sleep acts in concert with the central CS, but also independently towards an optimal internal temporal order (132). Specific sleep stages are closely related with specific clock gene expression in the SCN and are tightly ruled by the CS (81, 132, 258). SD has been associated with circadian-related gene expression alterations in humans (259–262). In addition, SD also relates to various HPA axis dysregulations (e.g., flattened CORT rhythm amplitude, blunted CORT awakening response (CAR), increased but also decreased diurnal CORT levels, higher CRH levels) and altered endocrine stress reactivity (e.g., attenuated pituitary ACTH reactivity, increased adrenocortical ACTH sensitivity) (257, 263–269), as well as to altered autonomic regulation with increased sympatho-adrenal and reduced vagal activity and blunted cardiovascular autonomic rhythmicity and autonomic reactivity (257, 270–273). Accordingly, chronodisruption in humans has been associated with increased risk for cardiovascular morbidity, metabolic consequences, inflammation, immune dysregulation, psychiatric disorders and even elevated cancer risk (226, 240, 274–280). Interestingly, even circadian gene polymorphisms have been associated with some similar consequences (281, 282).

Stress and Chronodisruption

In addition to other crucial circadian cues that can dysregulate circadian rhythms (e.g., SD, nutrition, light), stress can also lead to acute/reversible or sustained chronodisruption. Normally, after exposure to stressors, the SS can transiently override the CS creating a transient uncoupling of the central and peripheral circadian rhythm, through a hGR-related phase shift of peripheral clock-related genes (182, 197, 207, 212, 217, 221, 283). Thereby, the SCN is only indirectly influenced (198) and is, thus, able to maintain its master rhythm and restore its initial main phase to the periphery after stress termination (283, 284). Indeed, subacute stressors have been experimentally shown to have only transient impact on SCN-regulated rhythms in animal research (285, 286). However, the stability of the SCN clock appears to fade away after extensive acute or chronic physical, psychological, inflammatory, or metabolic stress (25). For example, in a study comparing single versus chronic social defeat across two weeks, single stress exposure advanced only the adrenal peripheral clock, while chronic stress also clocks in the CNS (287). Animal research provides additional evidence that chronic mild stress disrupts the regulated gene expression of several clock genes in several peripheral (287, 288), but also CNS tissues, including the hippocampus, amygdala, PFC (202, 286, 289, 290) and the SCN (283, 284, 291, 292). Chronic stress exposure in mice has been shown to alter the circadian properties of the HPA axis (293, 294), while extensive physical stress after surgery in humans leads to disturbances in MLT, CORT and core body temperature rhythms (295). In addition, numerous human and animal studies suggest that acute extensive and chronic stress can affect major sleep centers of the brain (202, 205, 288, 289, 293–296) and, thus, influence sleep physiology leading to both immediate and long-lasting sleep disruption (297–299).

Circadian-Phase-Dependent Stressor Effects

Apart from the physiological circadian activity of the SS, the stress responsiveness also displays diurnal sensitivity changes, probably through differential interference of the SCN to different brain areas (146, 159, 300). For example, acute psychological stress, involving higher brain areas and the limbic system, as well as acute physical external stress (i.e., restraint/immobilization, foot shock, shaking stress) exert the largest stress response during the rest phase (301, 302), when the HPA axis is less responsive, while acute physiological internal stress (i.e., oxidative stress, hypoglycaemia, hemorrhage), relayed to the PVN and brainstem, at the beginning of the activity phase (303, 304), when the HPA axis is most sensitive to stimulation (175). This appears reasonable, as acute physiological internal stress represents a greater threat during the active phase of animals, while acute external physical stressors (e.g., predator attack) during the inactive phase, while animals are asleep.

Interestingly, further experimental findings in animals suggest that repeated external stress exposure (i.e., chronic stress) has a more detrimental effect when applied during the inactive phase, (284, 305–308), while chronic psychosocial stress (i.e. social-defeat paradigm) shows inverse effects and exerts more detrimental effects during the active phase (307, 309) in animal research. These results jointly suggest that the effect of a stressor depends not only on the circadian phase of exposure, but also on the interaction of the circadian phase with the stressor type, as well as with the chronicity of the stressor (25, 310). For example, both physical and psychological stress at the beginning of the light phase leads to a phase advance, while at the beginning of the dark phase to a phase delay of PER2 expression in mice (286).

CHRONODISRUPTION AND TRAUMATIC STRESS

The stress-related effects on internal rhythms described above have supported a recent research focus on the potential causal role of SD and chronodisruption in the acute pathophysiology and the development of long-term effects of traumatic stress exposure, suggesting that chronodisruption may represent a potential underlying neurobiologic link (311–315). The association between sleep and circadian disruption and

psychopathology was first officially noted by Emil Kreapelin in 1883 (316) and has evolved through the years by numerous biological findings (317).

Traumatic stress exposure may cause both immediate and long-lasting SD (297–299), which may represent a central pathway mediating the enduring neurobiological correlates of trauma (297, 311, 312, 318–320) [cf. **Figure 6**]. For example, several human cohort studies have repeatedly suggested that early-life traumatic stress exposure is related to adult SD years later, including global (i.e., insomnia), but also other specific sleep pathologies, such as prolonged sleep onset latency, shortened total sleep time, decreased sleep efficiency, nightmare related distress, increased number of awakenings, sleep apnoea, and higher nocturnal activity (321–332). Such sleep dysregulation could further enhance maladaptive stress regulation and precipitate the neurobiological correlates of traumatic stress through impaired homeodynamic balance, resulting in the extensive symptomatology and comorbidity of trauma-related disorders (314, 333–350).

Posttraumatic Stress Disorder: When Time Stands Still

Posttraumatic stress disorder (PTSD) is classified in DSM-5 as a trauma- and stress-related disorder following a psychologically distressing event outside the range of usual human experience (351). Evidence of circadian dysregulation in PTSD mostly originates from sleep research findings. According to DSM-5, SD represents prominent clinical feature of the disorder with very high prevalence (312, 320, 351), and is often closely related to severity of PTSD psychopathology (352, 353) and resistant to first-line treatments (354–356).

SD in PTSD is associated with sleep-related arousal dysregulation (357) and include sleep avoidance, insomnia, nightmares, hyperarousal states, sleep terrors and nocturnal anxiety attacks, body-movement and breathing-related sleep disorders (311, 320, 358–362), with increased sympathovagal tone during rapid-eye-movement (REM) sleep, fragmented REM sleep patterns, and reduced REM theta activity (311–313, 318, 363–365). Similar findings have been in animal and human SD studies (366, 367). Interestingly, REM sleep disruption in the immediate aftermath of a trauma (311, 318, 319), as well as sleep impairment prior to traumatic stress exposure could represent risk factors for PTSD development (368, 369). SD prior to trauma have been specifically shown to be associated with a 2.5-fold increased risk of fulfilling PTSD criteria 3 months after a trauma in general population admitted to a hospital or after deployment in active military troops respectively (368, 369). SD after trauma thus represents a rather core than secondary feature of PTSD (297, 311, 312, 318–320, 370) and may be both a precipitating and perpetuating factor of the disorder (371–373).

Besides SD, traumatic stress also affects neural correlates of memory formation (374–376). Memory processing, formation and consolidation are directly influenced by sleep (377–387). Sleep promotes memory consolidation, particularly for emotionally salient information (383), while SD reduces the connectivity between amygdala and PFC (388) thus disrupting memory consolidation (389–393), as repeatedly shown in PTSD.

In addition to SD studies in PTSD, additional CS-related evidence on chronodisruption in PTSD originates from genetic, neuroendocrine, autonomic, and immune findings. For example, genome-wide association studies have also implicated to core circadian genes as PTSD candidate risk genes: pituitary adenylate cyclase-activating polypeptide (PACAP) and retinoid-related orphan receptor alpha (RORA-α) gene. PACAP is involved in phase resetting in response to light (394–396) and RORA-α is rhythmically expressed and regulates BMAL activity (397, 398). Furthermore, as immune system activity tightly follows circadian rhythms imposed by the SCN synchronisation (205, 399–405), our recent first report on the loss of the typical peripheral biphasic rhythm of IL-6 in combat stress exposed individuals (406), is of particular importance.

Further neuroendocrine findings in PTSD repeatedly show increased central CRH levels, altered HPA axis reactivity with enhanced negative feedback inhibition and blunted circadian CORT rhythm and CAR, while some studies—but not all—have shown decreased circulating concentrations of CORT (407–419). Similarly, patients with PTSD exhibit increased autonomic reactivity, elevated central and peripheral norepinephrine concentrations, higher basal heart rate, increased sympathovagal balance, blunted salivary alpha-amylase awakening response and, most importantly, blunted diurnal autonomic differences (341, 417, 420–427), suggesting central neuroautonomic dysregulation leading to higher cardiovascular risk in PTSD (415, 428, 429). In addition, disrupted MLT levels in the first 48 h after traumatic stress exposure were shown to be associated with a higher PTSD development risk (430).

Finally, PTSD has been frequently related to several other comorbidities, such as chronic fatigue syndrome (CFS) (431–434), fibromyalgia (435–439), rheumatoid arthritis (348), which all share a very similar underlying neuroendocrinological profile to PTSD (e.g., hypocortisolism, blunted diurnal CORT rhythm and HPA axis reactivity) (440–445) and have all been repeatedly associated with sustained chronodisruption (446–456).

CHRONOTHERAPEUTIC IMPLICATIONS FOR PTSD

Current evidence suggests that SD and CD may have a vital predispositional role in PTSD development (314), while their effective treatment could be associated with substantial improvement of overall PTSD symptomology (312, 457–459). Nevertheless, SD is still often clinically addressed as a secondary symptom in PTSD. Careful assessment and treatment of SD and CD should therefore be an integral part in PTSD management (356, 364, 371–373). Cognitive-behavioral sleep management in PTSD constitutes a widely acceptable and effective treatment option with durable gains and beneficial effects (356, 460–462). In addition, the antihypertensives α-1 adrenoreceptor antagonist prazosin and α-2 adrenoreceptor agonist clonidine, the synthetic cannabinoid receptor 1 and 2 agonist nabilone and the multilemodal antidepressant trazodone (i.e., serotonin-reuptake inhibitor, 5-HT_{2A} receptor agonist, histamine H1 receptor

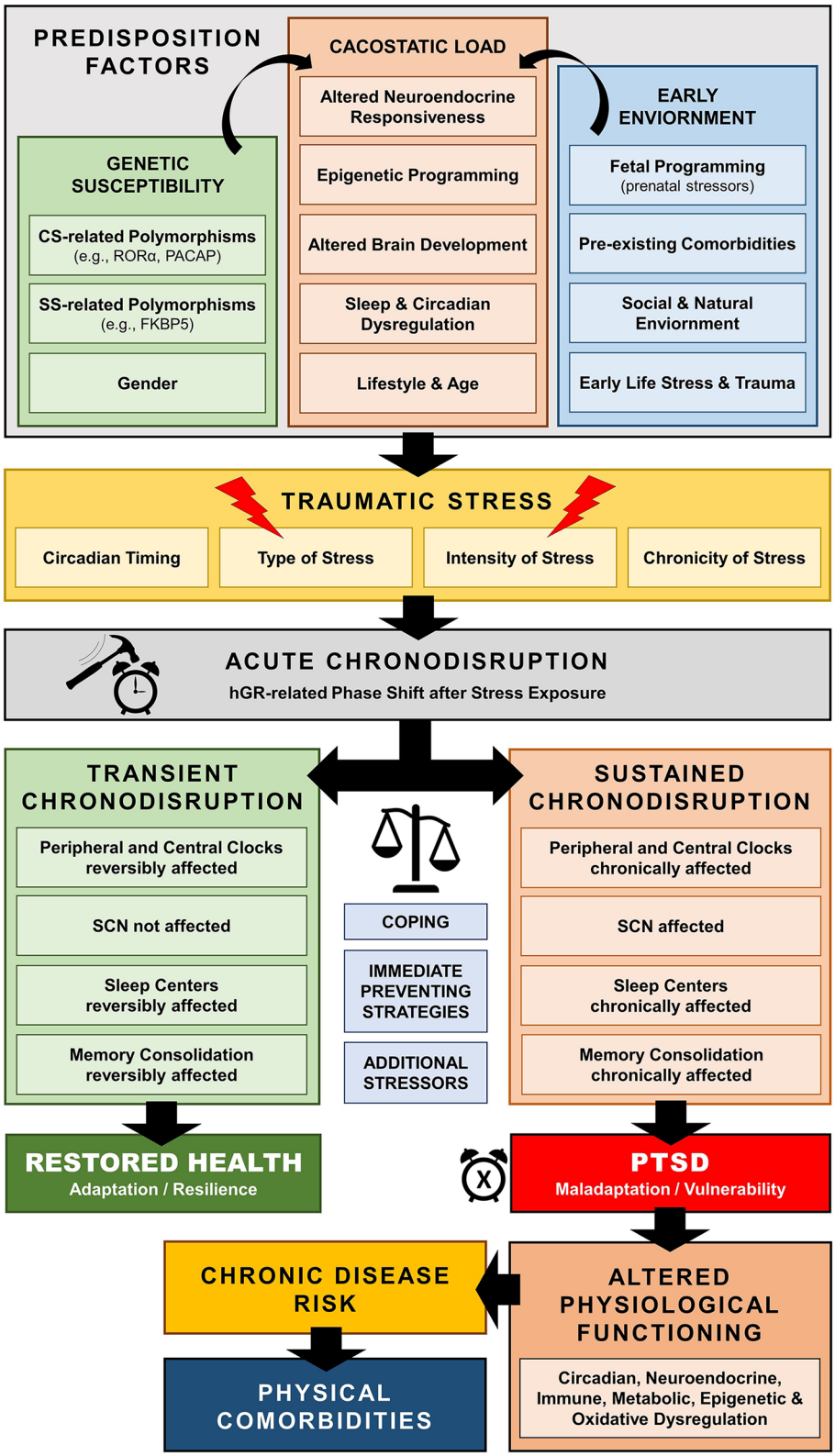

FIGURE 6 | Schematic model of trauma-related chronodisruption as underlying biological pathway leading to posttraumatic stress disorder (PTSD) and PTSD-related comorbidities.

antagonist, α-1 and α-2 adrenoreceptor antagonist) have been all shown to be effective pharmacological approaches for PTSD-related sleep disturbances and trauma-specific nightmares (463–467). Standard pharmacological sleep management in PTSD, however, may treat sleep quantity sufficiently, but often fail to improve daytime functioning and restore CD in PTSD (132, 468). Therefore, development of chronopharmacological interventions that would restore CS-related alterations and herethrough counteract changes in PTSD-related neurocircuitry could represent interesting novel therapeutic strategies (469–472).

Melatonergic Treatment

Recent experimental findings emphasize on a pleiotropic, but crucial role of MLT in mechanisms of sleep, cognition and memory, metabolism, pain, neuroimmunomodulation, stress endocrinology and physiology, circadian gene expression, oxidative stress, and epigenetics, thus suggesting a potentially beneficiary effect of an add-on melatonergic treatment in PTSD (374, 473). Numerous studies have repeatedly confirmed the efficacy of melatonergic treatment on almost every aspect of sleep disturbance, while preserving a benign side-effect profile and safety in both short- and long-term administration, with no efficacy wear-off, withdrawal effects or dependence risk (119, 474–480). MLT and melatonergic agonists, were found roboustly associated with (i) reduced sleep onset latency and increased sleep propensity, efficiency, quality, and total sleep duration in patients with insomnia, (ii) increased REM sleep percentage and continuity, normalization of sleep patterns, body-movement and breathing-related pathologies and improvements in subjective measures of daytime dysfunction in neuropsychiatric patients and (iii) advanced sleep/wake rhythm phase adjustment and sleep and wake-up propensity in healthy adults (119, 134, 135, 138, 474, 477, 478, 480–486). In addition, MLT is known to adjust and reset amplitude and phase of CNS (e.g., SCN, hippocampus, pituitary pars tuberalis) and peripheral (e.g., adrenal gland) circadian-related gene expression (172, 177, 178, 180, 487–489) and to moderate the circadian regulation of GR function (140, 141, 144, 183, 490). MLT also decreases hypothalamic CRH levels and inhibit the ACTH-stimulated CORT production in the primate and human adrenal gland (172, 177–180, 487–489), thus attenuating the adrenocortical secretory response in acute and chronic stress models (491–494). With respect to the ANS, MLT entrain disrupted autonomic rhythmicity by inhibiting central sympatho-adreno-medullary (SAM) outflow and shifting autonomic balance in favour of vagal activity (154, 495–498). Interestingly, research findings suggest a direct enhancing effect of melatonergic transmission in stimulus processing, memory consolidation, and conditional cued fear extinction, especially under stress (499–502). Finally, immediate melatonergic treatment directly after exposure to stress, normalizes the altered expression of Per 1 and Per 2 genes in hippocampal regions of rats, thus suggesting a possible immediate preventing properties (202). MLT has been shown to protect these hippocampal neurons from oxidative stress, by preventing GC-related toxicity through decrease of receptor translocation to nuclei in models of sleep deprivation and chronic stress (503–506). Taken together, MLT and melatonergic agents could therefore represent a promising adjuvant contribution to the clinical treatment and perhaps prevention of stress-related syndromes and comorbidities in mental disorders in general and PTSD in particular (124, 314, 471, 507–509).

Other Potential Treatment Possibilities

Further options for a pharmacological or nonpharmacological manipulation of the interplay between CS and SS in order to interfere in the pathophysiology of trauma-related disorders are of theoretical interest and deserve thorough further investigation through preclinical research and clinical confirmation. For example, exogenous application of GCs and GC-analogs in a time-of-day dependent fashion (i.e., as in immune therapy), could contribute to a reset of peripheral clocks (55, 144, 510) or even contribute to PTSD prevention if applied immediately after trauma exposure (511). On the other hand, pharmacological GR-antagonism has been found associated with insomnia symptoms improvement (512) and could also represent a potential approach.

As sleep promotes memory consolidation, particularly for emotionally salient information, sleep deprivation in the beginning of the resting phase directly after traumatic stress exposure may also decrease the risk of PTSD development (513), possibly through reduction of mPFC-amygdala connectivity (388, 390, 392). Furthermore, first findings suggest that casein kinase 1ϵ, a closely related clock components implicated in period determination, could represent a novel target of pharmacological inhibition, thus stabilizing the circadian clock against phase shift (472). Finally, it is important to mention, that selective serotonine reuptake inhibitors (SSRI), as first-line treatment option for PTSD, have been shown to exert additional, CS-related effects. In particular, fluoxetine treatment was shown to modulate the CS *via* phase advances of SCN neuronal firing (514) and also normalize disrupted circadian locomotor activity and hippocampal clock gene expression in a genetic mouse model of high trait anxiety and depression (515).

CONCLUSIONS

In Plato's cosmology, as presented in the Timaeus, time is suggested to depend on the periodic regularity of movement, which is secured and defined by the planets (516). This periodic movement of our planet has contributed to the evolution of the internal time-keeping system, that creates and maintains cellular and systemic rhythmicity, through temporal organization of physiologic processes throughout several structural levels in the organism, the CS. The intrinsic rhythmicity of this system is based on a core set of clock genes involved with an autoregulatory transcriptional/translational feedback loop machinery. By rephrasing Plato's words, we could, thus, state that human time depends on the periodic regularity of transcription, which is secured and defined by the clock genes. The award of the 2017 Nobel Prize in Physiology or Medicine to J.C. Hall, M. Rosbash and M. W. Young "for their discoveries of molecular mechanisms controlling the circadian rhythm" (517)

is a testament to the fundamental importance of circadian clocks and the molecular complexity of behavior regulation.

However, over the past seven decades, modern society has cultivated a new, round-the-clock lifestyle, which enhances temporal misalignment between internal (i.e., central and peripheral) and geophysical circadian cycles. Given the close interconnection between the CS and the SS at various levels, internal desynchrony could synergistically contribute to the development of a higher stress sensitivity and vulnerability for stress-related disorders. Understanding the mechanisms susceptible to chronodisruption following toxic stress exposure and their role in a chronically dysregulated circadian network in stress-related disorders could provide new insights into disease mechanisms, advancing psychochronobiological treatment possibilities and enabling preventive strategies in stress-exposed populations (74, 312, 518).

AUTHOR CONTRIBUTIONS

AA managed all literature searches. AA and NN wrote the first draft of the paper. VB, GC, and PP contributed with significant text passages and revised the draft for important intellectual content. All authors have contributed to, read and approved the final version of the manuscript.

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