EARLY LIFE ADVERSITY'S IMPACT ON BRAIN AND BODY: UNDERSTANDING DISRUPTED CIRCUITS TO IDENTIFY PREVENTIVE STRATEGIES

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ORIGINAL RESEARCH
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Childhood Trauma Affects Stress-Related Interoceptive Accuracy

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Early life adversity (ELA) may cause permanent disturbances in brain–body signaling. These disturbances are thought to contribute to physical symptoms and emotional dysregulation in adulthood. The current study investigated the effects of childhood trauma on young adults' interoceptive accuracy as an indicator of brain–body communication that may be dysregulated by ELA. Sixty-six participants completed an online questionnaire followed by a laboratory session including the socially evaluated cold pressor stress test during which ECG, salivary cortisol, and interoceptive accuracy were assessed. Childhood trauma was negatively related to interoceptive accuracy (IAc) after the stressor. This stress effect could not be observed for heart rate and cortisol, which were unrelated to IAc. Participants reporting higher baseline unpleasantness exhibited lower IAc after the stressor, while increases in unpleasantness due to the stressor were associated with higher IAc. Unpleasantness at baseline mediated the effect of childhood trauma on IAc after the stressor.

Keywords: early life adversity, childhood trauma, mental health, interoception, stress, unpleasantness

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INTRODUCTION

Childhood adversity can have substantial long-lasting consequences for the child concerned. Children who experienced traumatic events exhibit more mental and physical health problems in childhood and adulthood as compared to non-traumatized control participants (1–3). Existing research primarily focuses on trauma-related consequences for physical and mental health, whereas the psychophysiological mechanisms underlying these effects remain partially unclear. The current study focuses, therefore, on one candidate mechanism: the perception of bodily signals (i.e., interoception) and the potential relationship to childhood trauma.

The experience of traumatic stress during childhood can permanently alter stress responses (4–8). Chronic activation of the hypothalamic pituitary–adrenocortical (HPA) axis and the sympathetic–adreno–medullary (SAM) axis causes prolonged secretion of stress hormones that induce dysregulation of these interdependent stress axes (9), resulting in adverse effects on psychological and physical health (10). Dysregulation of the SAM axis—for example, may contribute to hypertension, whereas chronic activation of the HPA axis might result in hyper- or hyposecretion of cortisol, which is associated with major depression (11).

The activation of the physiological stress axes implies the efferent signal transmission from the brain to the body. It is likely that alterations in efferent brain–body communication also affect

afferent signals on the brain–body axes and, therefore, also their perception, i.e., interoception (7). Surprisingly, until now, it is unclear if dysregulation of the physiological stress axes, as has been previously documented following early life adversity (ELA), affects interoception.

Interoception is a major determinant of mental health. The objective performance in perceiving interoceptive sensations (e.g., heartbeats) is called interoceptive accuracy (IAc) (12) and is the central facet in contemporary models of interoception (13, 14). IAc is strongly associated with the experience and regulation of emotions: individuals with higher IAc show more negative affect after a stressor as compared to those with poorer habitual awareness of their bodily state (15), suggesting stronger emotional experience with higher IAc. This observation is in line with psychophysiological emotion theories (16, 17). Intense emotional experiences increase the need for emotion regulation (18), which may be one reason why IAc is positively related to emotion regulation (19). These findings illustrate the potential of IAc as protective factor against emotional disturbance. Altered interoception is observed in mental disorders with emotional disturbances and physical symptoms, such as depression and somatoform disorders (7, 20–25). Due to the association of IAc on emotional and physical well-being, it is critical to focus on stress as accelerant and regulator of bodily and emotional states and its important effects on physical and mental ill health (26).

IAc increases after an acute stressor if attentional resources are not compromised (i.e., no competition between interoceptive and exteroceptive signals; 7, 27), but it remains unclear, which stress axis contributes to this increase. More precisely, acute stress might result in increased IAc as increases in cardiac signals through activation of SAM axis ascend to the brain and are, therefore, more easily detectable (7, 28, 29). In addition, exogenous cortisol rapidly increases heartbeat evoked potentials (HEPs), which represent an indicator of cortical representation of interoceptive signals (30). One might expect, therefore, that an increase of IAc in response to an acute stressor may be due to increase in cortisol secretion as evoked by this stressor.

In contrast to acute stress, the relationship between physiological stress axes and interoception in chronic stress remains unclear. One might speculate that chronic activation of the SAM axis might result in a state of hyper-arousal (11, 31) that can dysregulate brain–body communication (7, 32, 33) and render it more difficult for the individual to attend to specific signals of the ascending signal stream due to a lower signal-to-noise ratio. Little is known about HPA axis activation and interoception. Effects of chronic stress on the HPA axis are manifold, including diurnal hyper- or hypo-secretion, blunted reactivity to acute stressors or feedback sensitivity (34). As of today, there are only two studies addressing cortisol and interoception: one reported higher HEPs after cortisol administration (30), whereas the other found a negative relationship between baseline cortisol and HEPs (35). Although these findings suggest that cortisol affects interoceptive signal processing in the CNS, the relationship of cortisol reactivity to an acute stressor and IAc remains unclear. This is, however, of particular relevance for health and disease, as cortisol reactivity is associated with stress-related disorders (36, 37) and could affect processing of bodily sensations, as well.

Another line of argumentation associating childhood trauma and IAc has been suggested recently by Oldroyd and colleagues (38), who specifically propose that interoception is affected by social interactions. Previous studies investigating the impact of absent parental responsiveness in infancy on dissociation in young adulthood (which includes emotional and physical detachments and should therefore inhibit interoception) found that childhood verbal abuse significantly predicts dissociation later in life (39). It is plausible, therefore, to assume an association between childhood trauma and interoception.

To date, there has—to our knowledge—not been any study investigating the effects of chronic and early life traumatic stress on IAc (7). However, due to its important implications in the development of bodily related mental disorders, it is crucial to understand if and how IAc is affected by early life stress. More insight into the relationship between IAc and ELA would help in the development of prevention programs to dismantle possible stress-induced dysregulation of IAc during childhood that might increase children's vulnerability to develop mental disorders later in life.

The aim of the current study was to investigate the impact of ELA on IAc and its relationship to acute stress reactivity. During the laboratory session, participants underwent a laboratory stress test. Autonomic stress response, salivary cortisol, and IAc were assessed at different time points before and after the stress test. We expected that (I.) IAc increases after the stressor (27). Additionally, we aimed to explore two related questions: (II.) what are the effects of ELA (i.e., childhood trauma) on IAc after an acute stressor? (III.) How is IAc related to stress-induced physiological (i.e., changes in heart rate (HR) and cortisol release) and (IV.) psychological changes (7, 15–16, 17, 28, 29).

METHOD

Participants

Sixty-six participants took part in this study. Forty-five were female, and 22 reported a parental divorce during their childhood. Mean age was 25 years (SD = 4.478). Most participants came originally from Luxembourg (N = 49) or Germany (N = 12). Two participants were born in Belgium, one in France, one in Russia, and one in South Korea. Educational background of the participants was high, with 30 having a university entrance diploma and 29 a university degree, 1 was still going to school, 1 had a certificate of secondary education, and 1 completed vocational training.

Questionnaire Measures

Childhood trauma was assessed using the German version of the Childhood Trauma Questionnaire (40). This 28-item questionnaire (five-point Likert scale ranging from 0 = not at all to 4 = very often) consists of five subscales: emotional abuse (α = .87), physical abuse (α = .87), sexual abuse (α = .98), emotional neglect (α = .88), and physical Neglect (α = .41). The CTQ has been shown to have good psychometric properties in previous research (40). In the current study, psychometric properties were also convincing (α = .71 for the global scale). The scores indicate

low to moderate childhood trauma scores in this sample, for the overall scale (M = 1.42, SD = 0.398), emotional abuse (M = 1.628, SD = 0.787), physical abuse (M = 1.075, SD = 0.263), sexual abuse (M = 1.088, SD = 0.489), emotional neglect (M = 2.012, SD = 0.819), and physical neglect (M = 1.319, SD = 0.449).

Psychophysiological Assessment

IAc was operationalized using the Schandry heartbeat perception task (41). Participants were asked to count their heartbeats over various periods of time (25, 35, 45, 55, and 65 s) that were presented in random order. IAc was calculated using the following formula:

$$IAC = \frac{1}{5}\sum_{k=1}^{5}\left(1 - \frac{\left(\left|no.of\ recorded\ heartbeats_k - no.of\ perceived\ heartbeats_k\right|\right)}{no.of\ recorded\ heartbeats_k}\right)$$

HR was used as an index of SAM axis activation and recorded continuously throughout the experiment. Electrocardiograph (ECG) signals were recorded using a precordial lead II electrocardiogram (RA, LL) with 1,000 Hz sampling rate, a hardware high-pass filter of 0.5 Hz, followed by a 0.5–35 Hz bandpass software filter. R-wave detection was automatically done in 1-min bins *via* the Mindware software's IMP 3.1.3 module (Lafayette, OH) and controlled *via* visual inspection for artifacts by the researchers afterwards. HR was averaged during a baseline period of 5 min at the beginning of the study (baseline period), during the 3 min of the tress induction and then again 15 min after the stressor for a period of 5 min, during which participants were instructed to sit still on their chair.

HPA-axis activity was assessed in terms of salivary cortisol reactivity. Saliva samples were collected using standard absorbent swabs (Salivette, Sarstedt; Nümbrecht, Germany) by participants. Immediately following completion of the experimental session, samples were frozen at −20°C. Salivary cortisol was assessed using a time-resolved immunoassay with fluorometric detection (42).

Acute Stress Induction

Acute stress was induced using the socially evaluated cold pressor test (SECPT) (43). An experimenter unknown to the participant entered the lab in a white lab coat and took a seat in front of the participant. He/she asked the participant to immerse their hand in a water container (water temperature 0–4°C) and to look into a video camera until requested otherwise. The participants were told that the experimenter and the camera would record their facial expression and behavior during the test. After 3 min,the test was over, and the experimenter left the room again with the video camera and the water container. Three participants of the 66 participants were excluded from analyses, as two did not keep their hand in the water for the duration of 3 min and 1 s because the water temperature was too high (i.e., 8°C).

Psychological Stress Response

Participants were asked to rate feelings of pain, anxiety, and unpleasantness on a visual analog scale (10 cm) before and immediately after the SECPT. Participants also completed the Self-Assessment Manikins (44) for arousal and mood valence right before and after the stress test. These emotional states are assessed using cartoon images that represent varying degrees of emotion intensities. Participants rated their current emotional state by selecting the box of the cartoon image that best represented how they felt at that moment (nine-point Likert scale). Psychometric properties of this inventory are convincing (44) and ratings on this scale have been shown to correlate with psychophysiological arousal states (i.e., skin conductance, corrugator, and zygomatic activity; 45).

Procedure

German-speaking participants were recruited online *via* social networks, through university postings and university circular e-mails. The study included a short online questionnaire and a 1-h experimental session. Participants were screened for the following exclusion criteria: cold intolerance (e.g., Raynaud's disease), current medication, alcohol consumption >30 g/day, illicit drug intake within the last 3 months, and current mental disorders that might affect the experimental results (e.g., depression, anxiety disorder, psychosis, suicidal ideation). More specifically, participants were asked during a telephone screening, if they had any current or past mental disorder, and if they had received any treatment for this condition (if any). A female experimenter was always present during the session for safety, sitting in a cubicle outside the visual field of the participant. After signing the informed consent, participants were attached to the physiological equipment and a 5-min baseline period (t1) ensued. Then, IAc was assessed, and psychological baseline measurements (arousal, mood valence, pain, and unpleasantness) were taken. This was followed by the SECPT. Ratings of arousal, mood valence, pain, and unpleasantness were again measured immediately after the SECPT. Salivary cortisol was collected immediately before the SECPT and then at 5, 15, 25, 35, and 45 min thereafter. IAc was measured again after the second saliva collection (approx. 6 min after SECPT onset). Participants received a financial compensation of 20 euros. The study design was approved by the Ethics Review Panel of the University of [Luxembourg].

Statistical Analysis

All data were scored and analyzed using *AcqKnowledge 4.2*, *Mindware 3.1.3*, and *SPSS 21*. Outlier identification was carried out by visual inspection for all variables, and extreme values (> 2.5 *SD*s above the mean) were set to missing. All missing data were independent of experimental condition and considered to be missing at random. Significance level was set at $p < .05$. Effect sizes are reported for any significant interaction or main effect using *Cohen's d* statistic (for *t*-tests) or partial eta-squared statistics (η_p^2; for ANOVA results). In the case of significant Levene-test results, *t*- and *F*-values for unequal variances are reported.

Firstly, three paired t-tests were calculated to investigate stress-induced changes of pain, anxiety, and unpleasantness ratings, respectively. Secondly, cortisol stress responses were analyzed by computing a repeated measures ANOVA using testing time (before the SECPT and then 5, 15, 25, 35, and 45 min after the SECPT) as within subject variable. Third, a repeated measures

ANOVA was conducted to investigate changes in HR in response to the SECPT, using testing time [baseline (t1), during the SECPT (t2), after the SECPT (t3), and at the end of the experiment (t4)] as within-subject variable.

Hypothesis (I.) was analyzed using a paired sample t-test comparing IAc before and after the SECPT.

To test hypothesis (II.), the data was restructured in long format (i.e., one time point per row) allowing for binary coded time [before (= 0) and after the SECPT (= 1)], childhood trauma, the interaction of time and trauma, and baseline IAc to be entered as predictors of IAc at the respective time points (before and after the stressor). Given the coding of time, the intercept represents the average IAc baseline score across participants. Because of the inclusion of baseline IAc, changes from pre- to post-SECPT (as indicated by the coefficient of time) are adjusted for differences in baseline IAc.[1]

Furthermore, to investigate if possible stress-induced effects could also be observed for changes in cortisol or heart rate, two exploratory regression analyses were calculated, respectively. Again, time was entered into the model, as was childhood trauma, the interaction of time and trauma, as well as baseline cortisol (or heart rate) as predictors for cortisol (or heart rate). All continuous variables were z-transformed before inclusion.

To investigate if IAc is affected by salivary cortisol (HPA axis) or HR (SAM axis) before, during, and after the acute stressor (III.), Pearson correlations between IAc and salivary cortisol and HR for all time points were calculated. We also analyzed if emotional reactivity was related to IAc (IV.) by calculating correlations between IAc and change scores in unpleasantness before and after the SECPT.

To examine the associations between childhood trauma, sex, interoception, unpleasantness, cortisol, and heart rate at baseline with IAc after the SECPT, we calculated a mediation model based on a bootstrapping procedure (46). Unpleasantness, cortisol, heart rate, and IAc at baseline were entered as mediators between childhood trauma and sex (i.e., male, female) and IAc after the SECPT with n = 10,000 resamples. Bias-corrected and accelerated 95% bootstrap confidence intervals (CI) for indirect effects were calculated.

RESULTS

Psychological Stress Response

Participants reported an increase in pain perception [$M_1 = 0.469$, $SD_1 = 0.858$, $M_2 = 5.231$, $SD_2 = 2.584$, $t(62) = -14.890$, $p < .001$, CI (−5.401, −4.123)], anxiety [$M_1 = 0.259$, $SD_1 = 0.496$, $M_2 = 0.549$, $SD_2 = 1.095$, $t(62) = -2.640$, $p = .008$, CI (−0.510, −0.071)], and unpleasantness [$M_1 = .754$, $SD_1 = 1.118$, $M_2 = 4.335$, $SD_2 = 2.708$, $t(62) = -9.610$, $p < .001$, CI [−4.326, −2.836)] after the SECPT as compared to baseline. Furthermore, stress-related increases in arousal [$M_1 = 4.698$, $SD_1 = 0.858$, $M_2 = 5.302$, $SD_2 = 1.931$, $t(62) = -3.677$, $p < .001$, CI (−.931, −.275)] and a decrease in positive valence [$M_1 = 7.127$, $SD_1 = 1.251$, $M_2 = 6.539$, $SD_2 = 1.389$, $t(63) = 3.801$, $p < .001$, CI (−.278,.896)] could be observed.

Stress Response

The main effect for testing time was significant [$F(1.534, 88.99) = 9.058$, $p = .001$, $\eta_p^2 = .135$; see **Figure 1**] suggesting a significant change of cortisol release over time. While *post hoc* tests showed a significant decrease of salivary cortisol from t1 to t2 [$t(59) = 3.339$, $p = .004$, $d = .10$, CI(.089;.367)], there was a significant increase from t2 to t3 [$t(59) = -2.591$, $p = .004$, $d = .349$, CI(−1.29; −.401)]. Cortisol levels were stable between t3 and t4 [$t(59) = 1.503$, $p = .552$, $d = .013$, CI(−.076;.343)]. Cortisol dropped significantly from t4 to t5 [$t(58) = 4.123$, $p < .001$, $d = .149$, CI(.224;.661)] and again from t5 to t6 [$t(59) = 4.516$, $p < .001$, d = .176, CI(.306;.815)].

The repeated-measures ANOVA of HR revealed a significant main effect for time [$F(1.819, 107.338) = 35.652$, $p < .001$, $\eta_p^2 = .377$; see **Figure 2**]. While HR did not differ between t1 ($M = 76.37$, $SD = 11.66$) and t2 [$M = 76.74$, $SE = 11.555$, $t(60) = .484$, $p = .630$, CI(−1.963; 1.198], there was a significant decrease in HR from t2 to t3 [$M = 71.78$, $SD = 11.329$, $t(60) = 6.522$, $p < .001$, CI(3.476; 6.522)]. HR did not change between t3 and t4 [$M = 71.92$, $SD = 11.873$, $t(59) = 0.469$, $p = .641$, CI(−.768;.476)].

Interoceptive Accuracy

IAc was higher after the SECPT (M = .768, SD = .20) compared to baseline levels [M = .721, SD = .20, t(62) = 3.039, p = .003, d = .235, CI(−.077; −.016)].

The regression analysis showed that changes from pre- to post-SECPT remained significant (β = .410, t = 2.958, p = .004) after adjustment for baseline differences in IAc and childhood trauma. Furthermore, IAc at baseline was a significant predictor for mean IAc (β = .889, t = 24.163, p < .001). The main effect for childhood trauma was not significant (β = −.005, t = −.096, p = .923), indicating no association between childhood trauma and IAc at baseline. There was a significant association, however, with time (β = −.305, t = −2.128, p = .035), indicating that higher levels of childhood trauma reduced the increase in IAc from pre- to

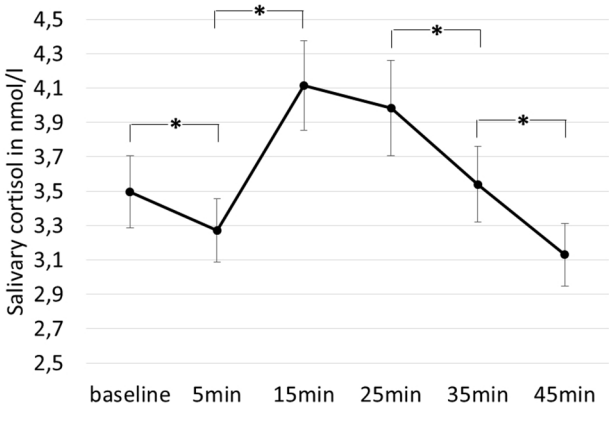

FIGURE 1 | Changes in cortisol over time (baseline = t1, 5 min = t2, 15 min = t3, 25 min = t4, 35 min = t5, 45 min = t6). Error bars indicate one standard error. Stars indicate significant differences between time points.

[1] The law of initial values predicts a systematic effect of baseline values on reactivity scores in that lower baseline scores are associated with larger increases to stress (i.e., negative correlation). It is, therefore, important to adjust change scores for baseline levels.

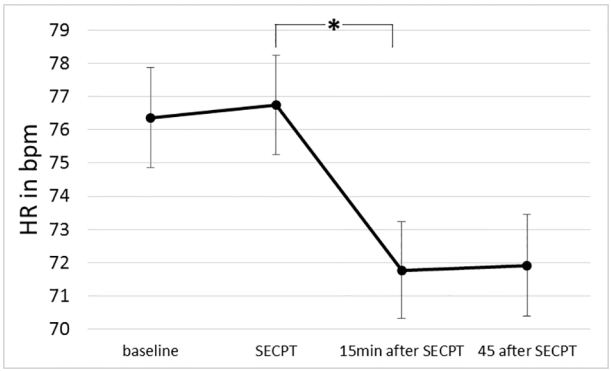

FIGURE 2 | Changes in HR over time (before, during, and after the stress). Error bars indicate one standard error. SECPT, socially evaluative cold pressor test. *indicate significant differences between time points.

post-SECPT (see **Figure 3**). **Figure 3** visualizes the association between time and IAc for different levels of childhood trauma (1 SD below the mean, mean, and 1 SD above the mean). An estimation of the region of significance by using the Johnson–Neyman technique (e.g., 47) indicated that, for participants scoring 1.263 standard deviations above the mean, no significant increase could be observed anymore from pre- to post-SECPT. These results even reveal a reverse relationship for childhood trauma scores 1.411 standard deviations above the mean, with post-SECPT scores being lower than pre SECPT scores.

We further analyzed if the effects observed for interoception were also reflected in physiological and hormonal stress-induced changes. We, therefore, replicated the same analyses using heart rate and cortisol instead of IAc as dependent variables in the regression analysis.

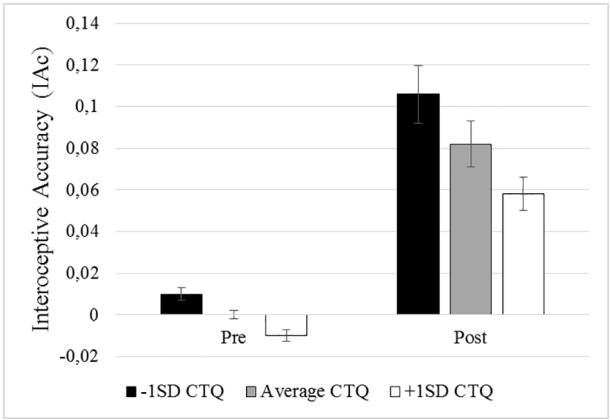

FIGURE 3 | Illustration of the interaction between time and trauma. The black column represents participants scoring 1 standard deviation above the mean scores of the childhood trauma questionnaire (CTQ), the gray column represents participants with mean CTQ scores, and the white column represents participants scoring 1 standard deviation below the mean of the CTQ. Time (pre = baseline; post = after the stressor) is illustrated on the x-axis, IAc on the y-axis.

We first included HR at baseline and HR during the stress induction in the model. HR was significantly higher during the SECPT than before (β = .245, t = 2.749, p = .048). The main effect of HR at baseline was significant (β = .938, t = 28.070, p < .001). Childhood trauma did not predict mean HR (β = .013, t = 1.329, p = .780), and the interaction between childhood trauma and time was only marginally significant (β = −.239, t = −1.889, p = .061).

Second, we included HR at baseline and HR 15 min after stress induction in the model. The main effect of HR at baseline was significant (β = .961, t = 50.958, p < .001). Heart rate was not significantly different 15 min after the SECPT compared to baseline (β = −.108, t = −1.563, p = .121), after adjusting for baseline HR levels and childhood trauma. Childhood trauma did not predict HR (β = .007, t = .259, p = .796), and also the interaction between childhood trauma and time was not significant (β = −.097, t = −1.356, p = .178).

We then entered cortisol at baseline (as predictor) and cortisol 5 min after the stress induction (as dependent variable) into the model. Cortisol was significantly lower directly after the SECPT than before (β = −.156, t = −2.120, p = .036). The main effect of cortisol at baseline was significant (β = .973, t = 48.679, p < .001). Childhood trauma did not predict cortisol levels (β = −.013, t = −0.446, p = .656), and also the interaction between childhood trauma and time was not significant (β = .080, t = 1.052, p = .295).

Finally, we entered cortisol at baseline and cortisol 15 min after stress induction into the model. Cortisol levels 15 min after the SECPT were not significantly different from cortisol levels at baseline (β = .077, t = 0.400, p = .690), after adjustment for cortisol baseline levels and childhood trauma. The main effect of cortisol at baseline was significant (β = .808, t = 15.526, p < .001). Childhood trauma did not predict cortisol (β = −.017, t = −0.226, p = .822), and also the interaction between childhood trauma and time was not significant (β = .036, t = 0.180, p = .858).

Interoceptive Accuracy and Stress-Induced Physiological and Psychological Changes

With regard to possible trauma-induced stress axis dysregulation, we calculated Pearson's correlations between salivary cortisol and HR before, during, and after the SECPT. None of these correlations was statistically significant ($p > .50$). Furthermore, we wanted to examine if stress-induced changes in unpleasantness during the SECPT were related to IAc after the stressor. Indeed, unpleasantness (IAc baseline: $r = -.294$, $p = .017$; IAc after the SECPT: $r = -.421$, $p < .001$) as well as stress-induced changes in unpleasantness (i.e., psychological reactivity; IAc after the SECPT: $r = .277$, $p = .025$) were related to IAc.

To further examine the mechanisms underlying these findings, we calculated a mediation model (see **Figure 4**). The results show that unpleasantness at baseline mediated the effect of childhood trauma on interoception after the stressor. Changes in unpleasantness, and unpleasantness after the stressor, no longer predicted interoception after the stressor, when entered into the model. Neither did cortisol nor heart rate after the

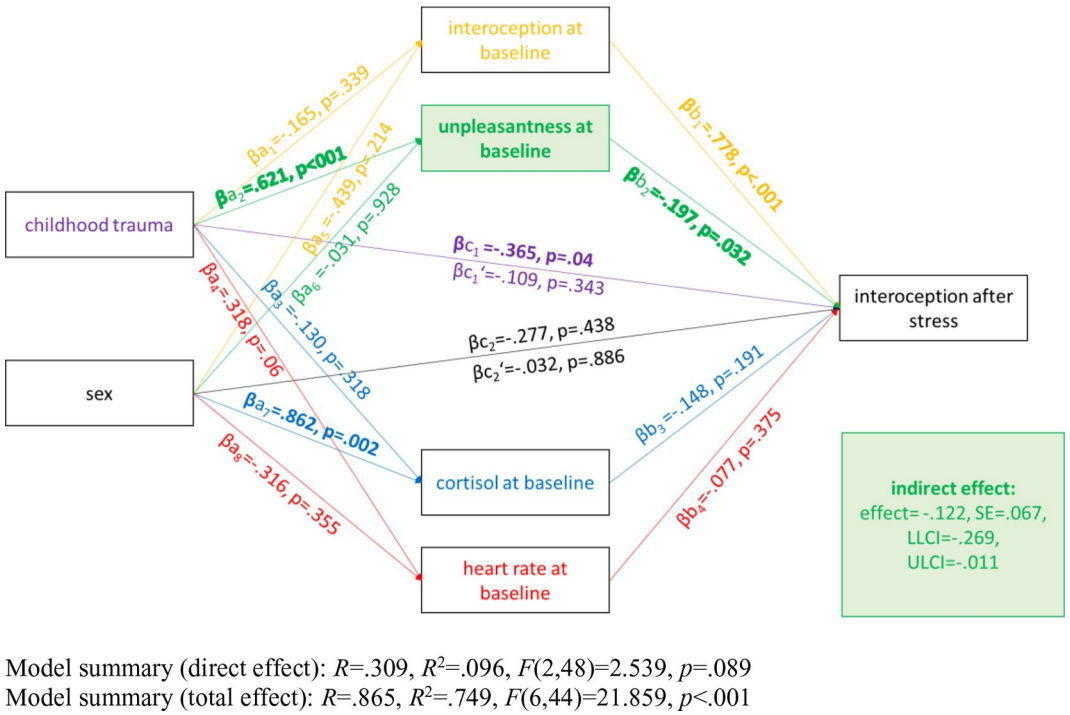

FIGURE 4 | Illustration of the bootstrapping analysis. Paths are represented using the following standardized coefficients: a_1 to a_8 indicate the coefficients from childhood trauma and sex to each mediator (i.e. interoception at baseline, unpleasantness at baseline, cortisol at baseline, and heart rate at baseline) respectively; b_1 to b_8 are the respective coefficients from the mediators (i.e., interoception at baseline, unpleasantness at baseline, cortisol at baseline, and heart rate at baseline) to interoception after the stressor; c is the coefficient that indicates the total effects of childhood trauma/sex on mental health symptoms without controlling for the mediators and c' reflects the path from childhood trauma/sex to interoception after the stressor controlling for the mediators (direct effects).

stressor predict interoception after the SECPT. The final model therefore only included baseline measurements.

DISCUSSION

The current study is the first to investigate the effects of traumatic childhood experiences on IAc before and after an acute stressor. This is of particular relevance, as early life stressors may affect brain–body communication, which is assumed to contribute to mental disorders associated with physical symptoms (e.g., 7, 20, 24, 25). After the SECPT, participants reported increases in pain, unpleasantness, and anxiety. Furthermore, cortisol significantly increased 15 min after the SECPT. HR dropped significantly 15 min after the stressor.

This study provides further evidence for the negative impact of childhood trauma on an individual's well-being in adulthood and suggests some potential mechanisms with regard to interoception. Childhood trauma significantly influenced IAc after the stressor: the more childhood trauma participants reported, the more difficult it was for them to perceive their heartbeat after the stressor. Interestingly, this stress effect could not be observed for our physiological measures (HR, cortisol) that were also not associated with IAc. One explanation for this pattern might be, therefore, that changes in IAc after an acute stressor are mainly a result of stress coping than of physiological changes. Interoception is theorized to be linked to the perception of emotional states and emotion regulation (15, 17, 19, 48) and—in this study—was related to unpleasantness rating. Participants reporting higher baseline unpleasantness showed lower IAc, while increases in unpleasantness due to the stressor lead to higher IAc. The latter finding is in line with study results from Kindermann and Werner (15), who found that individuals with higher IAc experienced more negative affect after an acute stressor. These opposing effects suggest trait and state differences in unpleasantness. Increases in unpleasantness might reflect stress-induced attentional shifts toward internal changes and therefore better sensitivity to physiological changes (e.g., increase in HR) facilitating perception of heartbeat (28, 49). The increased internal focus during stress may reflect an adaptive stress response, as it allows the assessment of bodily changes and stress-induced emotional states (17). This is supported by findings that the ability to correctly identify emotions is associated with higher IAc (50). The negative association between baseline unpleasantness and interoception is in line with studies observing a negative relationship between depression and IAc (51), as depressed individuals experience events as more unpleasant and report more negative and less positive affect during daily life (52). Baseline unpleasantness might represent a trait personality factor. Trait unpleasantness might, therefore, represent a chronic emotional state that is no longer informed by bodily signals. The mediation model indicates that the relationship between

childhood trauma and interoception might be fully mediated by unpleasantness at baseline with participants who experienced more childhood trauma reporting more trait unpleasantness and trait unpleasantness being negatively related to interoception after the stressor.

As the childhood trauma scores in the current sample were low to moderate, future studies should focus on severely traumatized populations to clarify if ELA may affect brain–body communication in these conditions. We could replicate previous findings in that IAc as measured with the Schandry paradigm increases after an acute stressor (27). Importantly, IAc after an acute stressor was negatively associated with childhood trauma, with participants scoring higher on the CTQ showing lower IAc than those with lower CTQ scores. This supports current models of chronic stress induced malfunctions of neural circuits underlining successful body–brain communication (7, 32, 33).

These results could, therefore, reflect the impact of high-intensity chronic stress on long-term changes in stress system functioning and trait emotionality (53). High-intensity, threatening, and chronic stresses may result in overwhelming physiological and cognitive reactions (53–56) that might not allow the individual to identify specific bodily signals. In line with this interpretation, reduced HEPs have been observed in individuals with borderline personality disorder (57), a disorder known to be highly associated with the experiences of traumatic events (58–60). Threatening situations overcharge an individual's coping abilities and consequently result in freezing and numbing (54, 55). Threat reactions might therefore result in suppression and denial of emotions and bodily symptoms as survival coping mechanism (54) and might explain why childhood trauma is associated with dissociation (that includes emotional and physical detachments and should therefore inhibit interoception) in later life (39) and, in this study, with reduced IAc after an acute stressor.

This is in line with Oldroyd and colleagues (38) who recently argued that social interactions can importantly affect interoception: while caregivers can—following their model—help the development of accurate interoception by validating their child's bodily experiences, they can also motivate children, through neglect and abuse, to avoid their bodily feelings. Neuroimaging studies provide evidence of shared neuronal regions of early attachment related experiences and interoception, such as the anterior cingulate cortex (61, 62) or the orbitofrontal cortex (63–67). While the orbitofrontal cortex, for instance, has not only been associated with early caregiving experiences and attachment (68), it has also been found to be implicated in the interpretation of bodily signals and affect regulation (68, 69). Indeed, attachment stiles are associated with interoception, as avoidant individuals reported lower interoceptive functioning (38). Furthermore, parental rejection of negative emotions was negatively related to the congruency of self-reported negative emotions and physiological distress signs (38). Since traumatic childhood experiences, such as emotional and physical abuse or neglect, are related to insecure attachment stiles that have protective value within the context of their families (70), one might expect an association between childhood trauma and interoception as well. This is corroborated by the current results, which lend further support to recent research relating early family experiences with interoceptive functioning (38, 39), while providing new knowledge on the association between childhood trauma and IAc after an acute stressor.

The finding of a negative association between childhood trauma and IAc after an acute stressor and its mediation through unpleasantness at baseline provides a theoretical foundation for prevention programs. It is important to help children to emotionally adapt to their traumatic experiences. Long-term changes in affect are often encountered after chronic stress and are even a diagnostic criterion for diagnoses such as post-traumatic stress disorder or personality change after catastrophic experience (71). It seems crucial to buffer the effect of childhood trauma on negative emotionality, by helping those experiencing childhood trauma to correctly integrate the traumatic experience into their biographic memory—for example, by using reframing techniques to reduce feelings of guilt, shame, and disgust. Additionally, techniques of enhancing non-evaluative mindful attention might help to enable children to better perceive their internal states. It seems important to help children who experienced childhood trauma to increase awareness to their stress-induced bodily and emotional changes over time and thereby enabling them to better differentiate cardiac signals from the stream of ascending bodily signals. This may have helped them to (a) better differentiate emotions from bodily changes and thereby to (b) better perceive and regulate their emotions.

LIMITATIONS

Limitations of the present study concern its cross-sectional design and the preponderance of students and female participants, which restricts the generalizability of the results to the general population. Questionnaire data such as childhood trauma was assessed retrospectively and might therefore be susceptible to memory bias. Future studies could use longitudinal designs to better understand the developmental trajectories leading to increased vulnerabilities for mental or physical disorders. Recently, potential shortcomings of the heart beat counting task have been discussed, such as low correlations between actual and perceived heart rates, or the influence of personal beliefs about one's heart rate and the ability of time perception on IAc (72, 73). Despite these potential shortcomings, the Schandry heartbeat perception task is a widely used, well-established measure in this field, whose validity was supported by a substantial overlap with neurophysiological indicators of interoceptive signal processing (i.e., heartbeat-evoked potentials) (74–77), as well as by a reduction in individuals with a degeneration of afferent autonomic nerves (78). Furthermore, it has been shown valuable in the identification of abnormal interoception in different mental disorders (20, 23).

CONCLUSION

This study reveals that childhood trauma is associated with lower IAc after an acute stressor, which may be explained by higher trait unpleasantness. The findings support current models of chronic stress induced malfunctions of neural circuits underlying successful brain–body communication. This finding may facilitate the development of prevention strategies targeting children who experienced childhood trauma with the aim to raise awareness to stress-induced bodily changes over time and thereby enabling them to better differentiate cardiac signals from the stream of ascending bodily signals. This may have helped them to (a) better differentiate emotions from bodily changes and thereby to (b) better perceive and regulate their emotions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was reviewed and approved by the Ethics Review Panel of the University of Luxembourg. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: VS, AS, MB, HS, CV. Formal Analysis: VS, JR. Funding Acquisition: VS, AS, GD, CV. Investigation: VS. Project Administration: VS, AS, CV. Visualization: VS, JR. Validation: AS, CV. Writing – Original Draft Preparation: VS. Supervision: AS, MB, GD, HS, CV. Writing – Review and Editing: VS, AS, JR, MB, CV. Methodology: VS, AS, MB, GD, CV. Resources: GD, CV.

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DNA Methylation in Healthy Older Adults With a History of Childhood Adversity—Findings From the Women 40+ Healthy Aging Study

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Background: Adversity in early development seems to increase the risk of stress-related somatic disorders later in life. Physiologically, functioning of the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes is often discussed as long-term mediators of risk. In particular, DNA methylation in the glucocorticoid receptor gene promoter (*NR3C1*) has been associated with type and strength of early life adversity and subsequent effects on HPA axis signaling in humans. Animal studies, moreover, suggest changes in DNA methylation in the estrogen receptor gene (*ER*α) upon early life adversity. We investigated the association of type and severity of childhood adversity with methylation in *NR3C1* and *ER*α and additionally considered associations between methylation and steroid hormone secretion.

Methods: The percentage of methylation within the *NR3C1* promoter and the *ER*α shore was investigated using dried blood spot samples of 103 healthy women aged 40–73 years. Childhood adversity was examined with the Childhood Trauma Questionnaire. Linear regression analyses were performed with methylation as dependent variable and the experience of emotional abuse and neglect, physical abuse and neglect, and sexual abuse (compared to non-experience) as independent variables. All analyses were controlled for age, BMI, annual household income, and smoking status and were adjusted for multiple testing.

Results: Overall, over 70% of the sample reported having experienced any kind of abuse or neglect of at least low intensity. There were no significant associations between childhood adversity and methylation in the *NR3C1* promoter (all $p > .10$). Participants reporting emotional abuse showed significantly higher methylation in the *ER*α shore than those who did not ($p = .001$). Additionally, higher levels of adversity were associated with higher levels of *ER*α shore methylation ($p = .001$).

Conclusion: In healthy women, early life adversity does not seem to result in NR3C1 promoter hypermethylation in midlife and older age. This is the first study in humans to suggest that childhood adversity might, however, epigenetically modify the ERα shore. Further studies are needed to gain a better understanding of why some individuals remain healthy and others develop psychopathologies in the face of childhood adversity.

Keywords: early life adversity, healthy women, methylation, *NR3C1*, *ER*α, cortisol, estradiol

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INTRODUCTION

Individuals with a history of abuse or neglect in early life are at risk of developing psychopathology later in life (1, 2). Such adverse experiences can have long-lasting effects on the physiological adaptation to stress and thus increase the risk of depression (3, 4), stress-related somatic disorders such as somatoform pain disorder and fibromyalgia (5), or posttraumatic stress disorder (PTSD; 6). Indeed, Kessler et al. (7) estimated that nearly 30% of all psychiatric disorders across countries can be traced back to childhood adversity. As women are generally at a higher risk of stress-related disorders than men (8, 9), women with a history of childhood adversity might represent an especially vulnerable group of individuals (10).

Early life stress can lead to a disruption in stress-sensitive systems such as the hypothalamic–pituitary–adrenal (HPA) axis. This axis is activated by acute stress and responds with a release of adrenal glucocorticoids such as cortisol into the bloodstream (11). Cortisol then exerts both enhancing and suppressing effects, depending on the target tissue (12). Once the stressor is withdrawn, the HPA axis down-regulates its own activity through a negative feedback loop, which is mainly mediated by binding of circulating cortisol molecules to glucocorticoid receptors (*GR*) in the hippocampus, hypothalamus, and the amygdala (13–15). While the human body is prepared to adapt upon acute stress, repeated or extreme stress exposure with insufficient or inaccurate adaptation can be damaging to health (16). Early life represents a sensitive time during which stress exposure can have long-lasting effects on the future stress adaptation (17). Previous studies found that individuals who had experienced childhood adversity showed a characteristic cortisol stress response. Specifically, moderate to severe childhood trauma and childhood emotional abuse were associated with a lower cortisol release in response to the dexamethasone-/corticotropin-releasing hormone (DEX/CRH) test (18, 19). Moreover, childhood physical abuse was associated with a blunted cortisol response to the Trier Social Stress Test (TSST), when compared to women without physical abuse (20). These findings suggest first a characteristic change in HPA axis signaling, which might result from different types of trauma and/or from a dose-response relationship, as different types of adversity regularly co-occur. Second, these effects were found not only in clinical samples but also in non-clinical samples and therefore suggest an effect of early life adversity on the HPA axis, which is independent of current psychopathology.

Among other mechanisms, early life adversity may lead to epigenetic DNA modifications in genes related to HPA axis signaling in order to promote adaptation to possible adversity later in life (21, 22 for reviews). Epigenetic DNA modifications can influence gene expression and behavior over a long period of time and therefore critically influence the health status of the entire organism (23). Methylation is thought to be the most stable epigenetic DNA modification and has therefore been studied in the context of the long-term effects of early life adversity on health and disease (24, 25).

Human studies suggest a causal role of early life adversity in methylation of the gene encoding the *GR* (mainly exon 1_F of the *NR3C1* gene promoter; 21, 26, 27). Higher methylation in hippocampal *NR3C1* lowers this specific gene expression and reduces the number of *GR*s. This leads to a diminished *GR*-mediated negative feedback loop and, in turn, to an exaggerated glucocorticoid secretion (28, 29). Postmortem analyses allow a direct investigation of hippocampal *NR3C1* methylation in humans. In a rare study in suicide victims, those who had experienced childhood abuse showed higher hippocampal *NR3C1* promoter methylation and a decreased $GR1_F$ expression compared to those without childhood abuse (30). It is difficult to disentangle the effects of early life adversity and current or past psychiatric disorder on methylation patterns, as disorders can themselves have effects on biomarkers (31). Therefore, the few studies in healthy adults with a history of childhood adversity are especially valuable for detecting whether methylation in *NR3C1* is responsive to early life adversity. In such adults, greater childhood adversity was associated with higher methylation in *NR3C1* (32, 33). These findings suggest that early life adversity might pose a significant independent risk factor for *NR3C1* methylation. Nevertheless, it should be mentioned that recent studies were unable to replicate this association (34, 35).

Possibly, *NR3C1* methylation affects HPA axis signaling in women to a greater extent than in men. First results show that, in otherwise healthy women, higher methylation in *NR3C1* was associated with higher cortisol secretion provoked by the TSST. In healthy men, by contrast, the *NR3C1* methylation and cortisol response were not associated (36). In line with these findings, healthy at high-risk men and women from the Dutch Famine Birth Cohort showed higher cortisol secretion in response to a psychological stress test, with higher methylation in *NR3C1*. The effect disappeared when controlling for sex (37). Circulating estradiol (E2) levels might mediate the association between *NR3C1* methylation and HPA axis response. As E2 levels are generally higher in women than in men, this would explain why women's HPA axis response is more sensitive to *NR3C1* methylation. More precisely, E2 takes effect upon binding to estrogen receptors (*ER*s). Two distinct forms of intracellular receptors mediate genomic effects of circulating estrogens: estrogen receptor alpha (*ER*α) and estrogen receptor beta (*ER*β) (38). Additionally, the G protein-coupled estrogen receptor (*GPER*) mediates rapid non-genomic effects of estrogens (39). *ER*s are abundantly expressed in cells of the hypothalamus, the pituitary, and the adrenal (39–41). All of these structures represent key players in HPA axis signaling, and E2 can therefore intervene with the negative feedback regulation of the HPA axis (reviewed in 42, 43, 44). Women accordingly show a slightly different stress response than do men (45, 46), which may be one of the reasons for women's pronounced risk of stress-related disorders (4, 47).

Animal studies suggest that the early social environment can influence *ER*α expression (48). In animal models, mothers represent the main source of social attachment. Early maternal care is assumed to influence the infant's *ER*α expression through its effects on DNA methylation in the *ER*α gene. Female rat offspring that received low maternal care in the form of licking and grooming (LG) were found to have a higher average methylation in the exon 1b *ER*α promoter region and lower *ER*α mRNA expression as adults than the offspring of high LG mothers (49, 50). It is suggested that, in particular, the imprint of

low maternal care should prepare the infant for a future hostile environment (51). Maternal care and *ER*β are assumed to be independent (50), while *GPER* has never been investigated in the context of maternal care. To conclude, similar to the effects on *NR3C1* methylation, negative early life experiences such as low maternal care can differentially modify methylation in the *ER*α gene in female animal models. To the best of our knowledge, it has never been investigated whether the early environment, and specifically the experience of early life adversity, may have a lasting effect on *ER*α gene methylation in women.

We hypothesized that women with a history of childhood adversity would show higher methylation in the *NR3C1* promoter and higher methylation in the *ER*α shore than women without such a history. Moreover, we hypothesized that the type of adversity would be differentially associated with methylation state and that greater adversity would be associated with higher methylation in both the *NR3C1* promoter and *ER*α shore. We expected that higher methylation in the *NR3C1* promoter would be associated with basal steroid hormone profiles, indicated by cortisol levels and the E2 to cortisol ratio (E2/C). Finally, from an exploratory perspective, we expected an association between *ER*α shore methylation and basal E2 levels and the E2/C ratio.

MATERIALS AND METHODS

The Current Study

The Women 40+ Healthy Aging Study was conducted at the University of Zurich and targeted subjectively healthy community-dwelling women between the age of 40 and 75. The present analyses are part of this large cross-sectional project, and the recruitment procedures are described in detail elsewhere (52).

Women reporting any acute or chronic somatic disease or mental disorder, or receiving any psychotherapeutic or psychopharmacological treatment during the last 6 months, were not included in the study. Moreover, habitual drinkers (more than two standard units of alcohol per day) were not included. Additional exclusion criteria were pregnancy (in the last 6 months), premature menopause or a surgical menopausal status (removal of either both ovaries or the uterus), intake of hormonal medication (oral contraceptives or hormone therapy in the last 6 months), shift work, and a recent long-distance flight. These criteria were in a first step assessed in an online self-screening and in a second step additionally confirmed by a trained study member in a telephone screening.

Study Procedures

Participants were invited to a weekday laboratory session at the University of Zurich between June 2017 and February 2018. Participants were asked to avoid any physical exercise for at least 24 h prior to the session and were instructed not to eat or drink (except for water) on the day of the session (53). All laboratory sessions were conducted one to one (one participant with one study member) and started at 7.45 a.m. with brief instructions. At 8.00 a.m., one saliva sample and several capillary blood spots were collected (54). On the day following the laboratory session, participants completed an online survey comprising validated psychological questionnaires. The procedures were controlled for menstrual cycle phase in women with menstrual bleedings (pre- and perimenopausal women), as defined by information on bleeding strength and patterns (55). Sample size calculations were performed using G*Power 3.1 (56). Specifically, calculations were based on F-tests using linear multiple regression analysis with a fixed model and investigating an R^2 increase. Under the assumption of a relatively small effect size ($f^2 = \cdot 15$; 57), we decided to collect data of 100 participants, yielding a power of around 0.95 to test the proposed hypotheses. The nominal alpha level of 0.05 was adjusted to.029 to take into account the number of hypotheses tested and the correlation between the predictors. Of the 130 women who completed the entire study, nine were not eligible for the data analyses due to medication intake prior to the laboratory session. The final sample size was therefore appropriate for the planned analyses. The study was conducted in accordance with the recommendations of the Cantonal Ethics Committee (KEK) Zurich, which approved the protocol. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Childhood Adversity

Childhood adversity was investigated with the German version of the Childhood Trauma Questionnaire (CTQ; 58). Using the CTQ, a sub-form distinction was made between adverse childhood experiences in the form of physical, emotional, and sexual abuse and adverse childhood experiences in the form of physical and emotional neglect. Additionally, a maltreatment score was calculated by summing up all individual categories of childhood adversity exceeding a critical threshold defined by Walker et al. (59). Critical thresholds were set as follows: physical abuse (8 points), emotional abuse (9 points), sexual abuse (6 points), physical neglect (8 points), and emotional neglect (10 points) (60). Higher maltreatment values indicate more exceeded thresholds, with values ranging from (0) *no threshold exceeded* to (5) *five thresholds exceeded*.

Methylation

DNA Extraction

Cytosine methylation was assessed from dried blood spot (DBS) DNA samples, as previously described elsewhere (see, e.g., 61, 62, 63). The Qiagen QIAamp DNA Investigator Kit (Qiagen, Hombrechtikon, Switzerland) was used to extract genomic DNA from three punches of blood-soaked filter paper (each 3 mm in diameter). Punches were then eluted in 30 µl of RNase-free water, and the DNA concentration was assessed using the Qubit Fluorometer (Thermo Fischer Scientific, Reinach, Switzerland). A total range in DNA from 41 to 168 ng was detected.

NGS Library Preparation

First, we performed bisulfite conversion of DNA using the EZ 96-DNA Methylation-Gold Kit (Zymo Research, Luzern, Switzerland). DNA was eluted in 20 µl of RNase-free water. The sequences of interest (*ER*α shore of promoter C: (hg 38) chr6:151,805,523-151,805,822 and *GR* promoter: (hg 38) Chr5:

143404021-143404338 were amplified using the following primers: *ER*α—frw 5'-GTTTTTTGTGAGTAGATAGTAAGTT-3' and rws: 5'-AAACCTACCCTACTAAATCAAAAAC-3,' *GR*: frw 5'-TTG AAG TTT TTT TAG AGG G-3' and rws 5'-AAT TTC TCC AAT TTC TTT TCT C-3' with the following thermocycler conditions: 95°C for 3 min, [98°C for 20 s, 58°C (*ER*α); 60°C (*NR3C1*) for 15 s, 72°C for 15 s] x 40, and a final step with 72°C for 45 s. Forward and reverse primers included universal primer sequences CS1/ CS2 (Fluidigm, California, USA) on 5.' The generated amplicons were then purified using the E-Gels 2% size selection technology (Thermo Fisher Scientific, Reinach, Switzerland). Indexing with unique single barcode (Fluidigm, California, USA) was then performed through a second PCR [95°C, 3 min; (98°C, 20 s; 60°C, 15 s; 72°C, 15 s) x 10; 72°C 45 s] on the purified amplicons. Samples were pooled and diluted (10x). The two libraries (*ER*α and *NR3C1*) were quantified using the Agilent 2200 Tape Station Instrument (Santa Clara, CA, USA) with HS DNA 1000 reagents. The two libraries (2 nM each) were merged, and sequencing was performed on the Illumina MiSeq sequencer using the V3, 600 cycles kit.

Interrogation of CpG Sites in the Targeted Amplicon

Quality analysis of adaptor sequences and bases was performed using Trimmomatic v0.35 (64). Low-quality products were removed according to the default settings. Bismark software (v0.19.0) was used to extract the counts of methylated (cytosines) and unmethylated (thymine) bases. Unmethylated and methylated counts were summed up, and CpG sites scoring less than 100 counts were removed in line with Chen et al. (65). Finally, methylation percentages were calculated by dividing unconverted counts by the total number of counts (methylated and unmethylated). To represent overall methylation in the *NR3C1* promoter region, one mean % methylation score across all 39 investigated CpGs of interest was created. Single CpG sites are presented in **Figure 1** and were chosen in line with Palma-Gudiel et al. (21). A similar approach was applied to quantify methylation in the *ER*α shore of promoter C (66). Again, methylation across the nine investigated CpGs (see **Figure 2**) was represented by a mean % score across all sites. As previous studies have investigated the association of childhood adversity and single CpGs in the *NR3C1* promoter, these associations were also tested and the results can be found in the supplementary material.

Steroid Hormone Levels

Saliva samples were assessed to analyze levels of E2 (pmol/L) and cortisol (nmol/L). All saliva samples were collected in 2-ml SaliCaps (IBL International GmbH, Hamburg, Germany) using the passive drool method. Saliva samples were stored at −20°C until biochemical analyses were performed. Thawed saliva samples were centrifuged and analyzed using enzyme-linked immunoassays (IBL International GmbH, Hamburg, Germany). Intra- and inter-assay variations were below 10%. Sensitivity was 1.10 pmol/L for E2 and 0.03 nmol/L for cortisol. The biochemical laboratory of the Department of Psychology, Clinical Psychology, and Psychotherapy at the University of Zurich performed the salivary analyses.

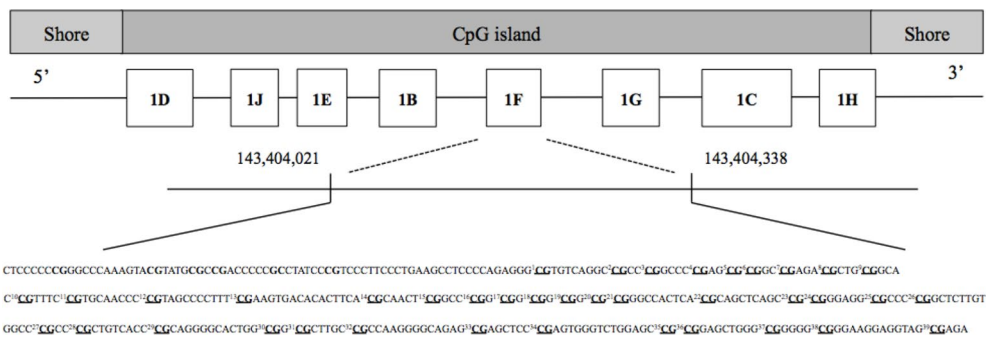

FIGURE 1 | Investigated single CpG sites in the glucocorticoid receptor (*GR*) gene promoter (*NR3C1*). Underlined CpGs represent the 39 single targeted sites. For analysis, one mean % methylation score across all 39 investigated CpGs of interest was created to represent overall methylation in the *NR3C1* promoter region.

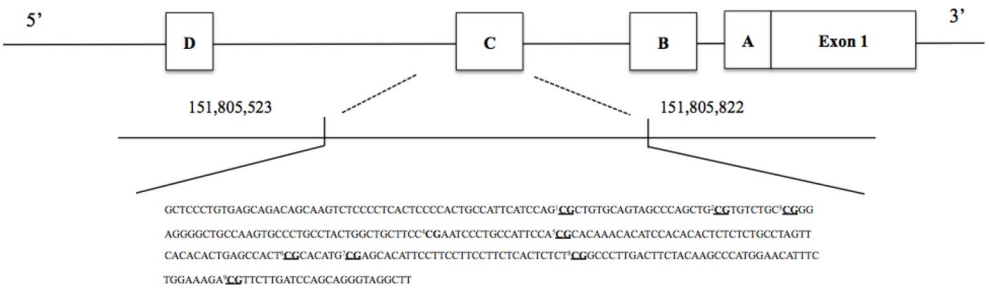

FIGURE 2 | Investigated single CpG sites in the estrogen receptor alpha shore (*ER*α). Underlined CpGs represent the nine single targeted sites. To represent overall methylation in the *ER*α shore, one mean % methylation score across all nine investigated CpGs of interest was created.

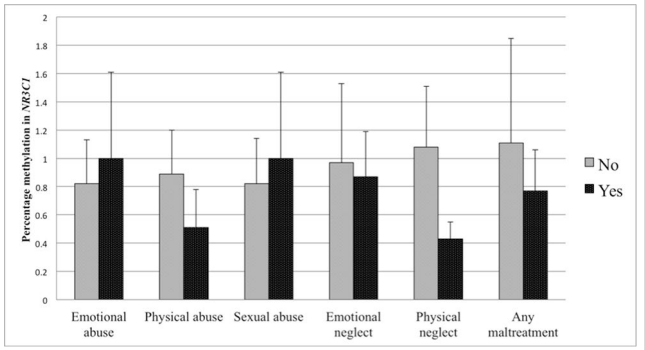

FIGURE 3 | Percent methylation in the glucocorticoid receptor (*GR*) gene promoter (*NR3C1*) in women with experience of specific sub-forms of abuse and neglect (black bars) compared to women without such experiences (grey bars). Bars represent mean values in *NR3C1* methylation and whiskers represent standard errors.

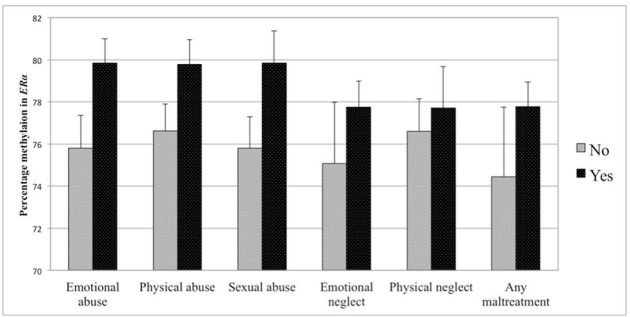

FIGURE 4 | Percent methylation in the estrogen receptor alpha gene shore (*ERα*) in women with experience of specific sub-forms of abuse and neglect (black bars) compared to women without such experiences (gray bars). Bars represent mean values in *ERα* shore methylation and whiskers represent standard errors.

Control Variables

Based on initial correlation analyses, all subsequent analyses were controlled for age, BMI, and socioeconomic status. Additionally, smoking status (in package-years) was controlled for due its known effect on methylation levels. Analyses involving steroid hormone levels were additionally controlled for the stress level in the week prior to the laboratory session, as assessed with the German version of the Perceived Stress Scale (PSS-10, 67).

Statistical Analyses

First, based on age and menopausal stage, two E2 values (22.7; 25.75 pmol/L) were considered as implausibly high and therefore excluded from the analyses. Additionally, two cortisol values (morning level: 86.93; 8.00 a.m. level: 46.77 nmol/L) and three cases of *NR3C1* methylation (30.57; 38.06; 55.85%) were excluded from further analyses. This decision was based on the comparison of single values with information from previous studies (21, 68). For cortisol, reports of recent stressful experiences or lack of sleep, which may provide an explanation for considerably higher than average values, were additionally considered. Methylation data in the *NR3C1* promoter were highly left-skewed and therefore log-transformed to approach normal distribution. Additionally, steroid hormone data were log-transformed. There were missing CTQ data from 16 participants, who were therefore excluded from further analyses. To test the association between type of adversity and methylation, partial correlations between adversity sub-forms and methylation in the *NR3C1* promoter and the *ER*α shore were calculated. The association between strength of adversity and methylation was tested using partial correlations between the maltreatment score and methylation in the *NR3C1* promoter and the *ER*α shore. Finally, the association between methylation and steroid hormone levels was investigated using partial correlations. In an additional step, the results of the correlation analyses were verified using stepwise linear regression analyses including control variables (step one) and independent variables (step two). The analyses were adjusted for multiple testing. As proposed by Benjamini and Hochberg (69), the α-value of 0.05 was adjusted (multiplied) by (n+1)/2n (whereas n = 6). This adjustment was performed to account for the inter-dependence of the six CTQ sub-forms including the maltreatment score. Therefore, an α-value of $p < 0.029$ was considered statistically significant. For analyses regarding the association between methylation and steroid hormones, the level of statistical significance was set at $p < .05$. Analyses were performed using SPSS (version 23, IBL).

RESULTS

Demographic Characteristics

Table 1 summarizes the general sample characteristics of the entire study population. There were no differences between women with any kind of abuse or neglect and women without any such experience with regard to marital status ($p = .74$), education ($p = .35$), smoking status ($p = .95$), age ($p = .14$), and BMI ($p = .16$). Women without experience of childhood abuse had a slightly, although not significantly ($p = .067$), higher annual household income than those who had experienced such abuse. Moreover, women with a history of childhood adversity showed a significantly lower E2/C ratio than did women without such a history ($p = .039$; see **Table 2**).

Prevalence of Childhood Adversities

The prevalence rates of the sub-forms of abuse and neglect are depicted in **Table 3**. Overall, 70.6% of the sample reported having experienced any kind of abuse or neglect of at least low intensity.

Childhood Adversity and Percent Methylation

The first set of analyses tested the association of sub-forms of childhood adversity with methylation. There was no association of any childhood adversity sub-form with methylation in the *NR3C1* promoter when controlling for age, BMI, household income, and smoking status (see **Table 4**). Controlling for the same variables, methylation in the *ER*α shore was, however, significantly positively

TABLE 1 | Descriptive statistics of the sample.

	N	%
Country of origin		
Switzerland	105	88.2
Germany, Austria, Liechtenstein	13	10.9
Hungary	1	0.9
Marital status		
Single	28	23.5
Married	63	52.9
Divorced	22	18.5
Widowed	6	5.0
Education		
Vocational education	42	35.3
High school-leaving certificate	23	19.3
College/University degree	53	44.5
Other	1	0.8
Smoking		
Yes	10	8.4
1–2 (cigarettes per day)	6	5.0
3–10 (cigarettes per day)	2	1.7
>10 (cigarettes per day)	2	1.7
	Mean	**SD**
Type of adversity		
Emotional abuse	8.64	4.11
Physical abuse	5.87	1.89
Sexual abuse	6.06	2.54
Emotional neglect	12.25	4.36
Physical neglect	7.58	2.96
Maltreatment score	1.99	1.69

associated with physical neglect. The associations between different sub-forms of adversity and methylation are depicted in **Figure 3** for the *NR3C1* promoter and **Figure 4** for the *ER*α shore, revealing differences in methylation scores as a function of the experience *vs*. non-experience of the different sub-forms of childhood adversity.

In a next step, regression analyses were performed to test whether the experience compared to non-experience of childhood adversity (dummy-coded sub-forms) was predictive for the methylation in the *NR3C1* promoter. In a first step, control variables were accounted for (R^2 = .04), while adding any of the childhood adversity sub-forms did not significantly improve the model fit (all $p > .10$). Hence, differences in *NR3C1* promoter methylation could not be explained by childhood adversity.

The same set of regression analyses was repeated with methylation in the *ERα* shore as dependent variable. After taking the control variables into account in a first step (R^2 = 13.0), the results revealed that adding any kind of maltreatment (ΔR^2 = .037, p = .030), emotional abuse (ΔR^2 = .089, p = .001), physical abuse (ΔR^2 = .027, p = .067), sexual abuse (ΔR^2 = .019, p = .106), or emotional neglect (ΔR^2 = .036, p = .040) improved the model fit. After controlling for multiple testing, only emotional abuse was retained as a statistically significant predictor of methylation in the *ER*α shore (see **Table 5**).

Additive Effects of Childhood Adversities on Percent Methylation

Partial correlations revealed that methylation in the *NR3C1* promoter was not associated with the score for reported maltreatment when controlling for age, BMI, annual household income, and smoking status. This lack of association was additionally confirmed in linear regression analyses. In a first step, control variables were accounted for (R^2 = .04), while adding the maltreatment score did not significantly improve the model fit ($p > .10$). The level of maltreatment was therefore not predictive for methylation in the *NR3C1* promoter. The maltreatment score was, however, significantly positively associated with methylation in the *ERα* shore. The second regression analysis therefore tested whether the maltreatment score was predictive for the methylation level in the *ER*α shore. After taking the control variables into account in a first step (R^2 = 13.0), the results revealed that adding the maltreatment score significantly improved the model fit (ΔR^2 = .094, p = .001). A higher maltreatment score was associated with higher *ER*α shore methylation, even when controlling for multiple testing (see **Table 5**). The association between the maltreatment score and methylation levels is illustrated in **Figure 5** for the *NR3C1* promoter and **Figure 6** for the *ER*α shore.

Percent Methylation and Steroid Hormone Levels

Finally, the association between methylation and circulating steroid hormone levels was investigated. Methylation in the *NR3C1* promoter was negatively associated with E2 and the

TABLE 2 | Descriptive statistics representing mean values of the total study sample and mean values based on participants' history of childhood adversity.

	Total mean (SD)	With adversity mean (SD)	Without adversity mean (SD)	p-value
Control variables				
Age	53.37 (8.98)	54.00 (8.86)	51.00 (9.19)	.138
Annual household income (CHF)	127,874 (75, 505)	121,329 (71, 889)	152,479 (84, 884)	.067
Body mass index (kg/m^2)	23.03 (3.96)	23.27 (3.82)	22.11 (3.01)	.163
Percent methylation				
NR3C1 promoter	.85 (2.78)	.77 (2.44)	1.11(3.72)	.60
ERα shore	77.11 (12.32)	77.78 (11.20)	74.44 (16.07)	.25
Steroid hormones				
Estradiol (pmol/L)	6.15 (5.22)	5.79 (5.00)	7.54 (5.84)	.137
Waking cortisol (nmol/L)	7.05 (10.40)	5.54 (4.87)	7.30 (6.52)	.145
08.00 a.m. cortisol (nmol/L)	6.64 (5.73)	6.37 (4.36)	6.05 (4.55)	.754
Estradiol/cortisol ratio	1.36 (1.43)	1.22 (1.56)	1.90 (2.13)	.039

CHF, Swiss Francs. Statistics represent two-tailed comparisons of unstandardized mean values between women with vs. without a history of childhood adversity.

TABLE 3 | Classification of traumatic childhood events with the Childhood Trauma Questionnaire (CTQ) sub-forms.

	None (or minimal)	Low (to moderate)	Moderate (to severe)	Severe (to extreme)	No information
Emotional abuse	82 (67.6%)	17 (13.9%)	12 (10.0%)	4 (3.4%)	6 (5.1%)
Physical abuse	103 (85.1%)	7 (5.7%)	4 (3.4%)	2 (1.7%)	5 (4.1%)
Sexual abuse	92 (75.7%)	18 (15.0%)	5 (4.2%)	4 (3.4%)	2 (1.7%)
Emotional neglect	36 (30.3%)	26 (21.3%)	42 (34.6%)	8 (6.5%)	9 (7.3%)
Physical neglect	68 (55.9%)	23 (18.9%)	13 (10.7%)	6 (5.5%)	11 (9.0%)

Values represent numbers of participants (N) and percentage of the entire sample.

TABLE 4 | Associations between Childhood Trauma Questionnaire sub-forms and percent methylation.

	1	2	3	4	5	6	7	8
Log *NR3C1* promoter (1)	1.00	.07	−.08	−.03	.06	−.04	−.02	−.02
*ER*α shore (2)		1.00	.16	.18	.05	.11	.24*	.28**
Emotional abuse (3)			1.00	.75***	.21	.66***	.63***	.77***
Physical abuse (4)				1.00	.24*	.43***	.69***	.58***
Sexual abuse (5)					1.00	.19	.20	.30**
Emotional neglect (6)						1.00	.49***	.78***
Physical neglect (7)							1.00	.66***
Maltreatment score (8)								1.00

*Statistics represent partial correlations (r) controlled for age, body mass index (kg/m2), annual household income, and smoking status. p < .029 = * (adjusted for multiple testing), p < .01 = **, p < .001***.

TABLE 5 | Linear regression analyses with *ER*α shore methylation as dependent variable and type and severity of childhood adversity as independent variables.

Severity and type of adversity	Adjusted b (95% CI)	p-value
Type of adversity		
Any childhood adversity	.20	.030
Emotional abuse	.32	.001
Physical abuse	.17	.067
Sexual abuse	.15	.106
Emotional neglect	.20	.040
Physical neglect	.11	.251
Severity of adversity		
Maltreatment score	.33	.001

Values on type of adversity represent dummy-coded variables for experience of this type of adversity (non-experience represents the reference categories). Analyses are adjusted for age, BMI, annual household income, and smoking status. p < .029 is considered as statistically significant (adjusted for multiple testing).

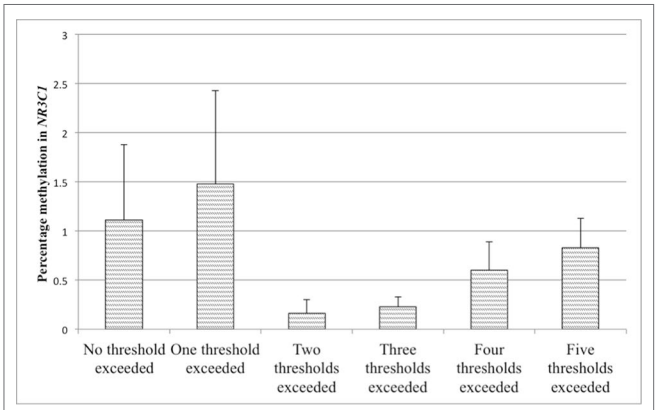

FIGURE 5 | Values represent means and standard errors (SEM) of percent methylation in the glucocorticoid receptor (GR) gene promoter (*NR3C1*). A higher maltreatment score reflects a higher number of categories of abuse or neglect above a critical threshold.

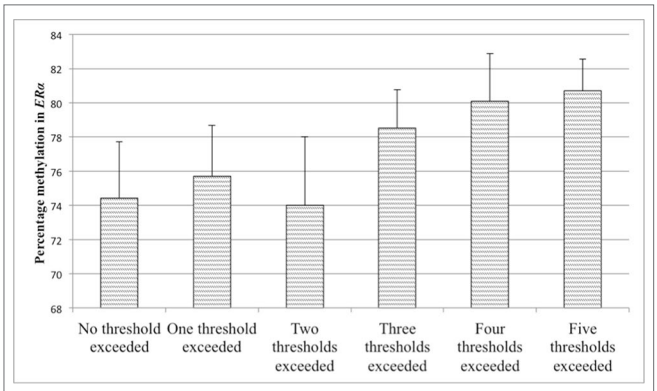

FIGURE 6 | Values represent means and standard errors (SEM) of percent methylation in the estrogen receptor alpha gene shore (*ER*α). A higher maltreatment score reflects a higher number of categories of abuse or neglect above a critical threshold.

E2/C ratio, although these associations did not reach statistical significance after adjusting for the control variables age, BMI, annual household income, smoking status, and acute stress ($p < .10$). Methylation in the *ER*α shore was significantly positively associated with E2 and positively, although not significantly, associated with the E2/C ratio ($p < .10$; see **Table 6**).

DISCUSSION

In this study, the effects of type and strength of early life adversity on the *NR3C1* promoter and the *ER*α shore methylation and subsequent steroid hormone levels were investigated in a sample of healthy middle-aged to older women. A large proportion of the sample (60%) reported emotional neglect of at least minimal intensity, with more than 40% reporting moderate or severe levels. Almost 40% reported at least minimal physical neglect, while rates for emotional abuse, sexual abuse, and physical abuse were considerably lower. As expected for a community-dwelling sample, prevalence rates for severe forms of adversity were rather low (70). Moreover, sub-forms of abuse and neglect showed high

TABLE 6 | Associations between percent methylation and steroid hormone levels.

	1	2	3	4	5	6
Log *NR3C1* promoter (1)	1.00	.11	−.17	−.09	.10	−.17
*ER*α shore (2)		1.00	.21*	.10	−.02	.17
Log Estradiol (3)			1.00	.14	−.01	.78***
Log Walking Cortisol (4)				1.00	−.11	.12*
Log 8:00 am Cortisol (5)					1.00	−.64***
Log Estradiol/ Cortisol ratio (6)						1.00

*Statistics represent partial correlations (r) controlled for age, body mass index (kg/m2), annual household income, and smoking status, and acute stress. p < .05 = *, p < .001***.*

intercorrelations. Contrary to our initial assumptions, methylation in the *NR3C1* promoter was neither associated with the type nor with the strength of adversity. As proposed, the experience of adversity was in turn associated with higher methylation in the *ERα* shore, with indications of a dose-response relationship. Furthermore, higher methylation in the *ER*α shore was associated with higher E2 levels and a higher E2/C ratio. Finally, higher *NR3C1* promoter methylation was associated with higher basal cortisol levels and a lower E2/C ratio. All women in our study considered themselves as healthy, independent of a history of childhood adversity. This fact needs to be considered when interpreting the findings.

At first glance, the absence of an association between *NR3C1* promoter methylation and early life adversity seems to contradict the existing literature. Since the initial animal-based study by Weaver et al. (71), many human studies have been able to replicate a hypermethylation in *NR3C1* in children and adults with diverse pathological states who had experienced childhood adversity (i.e., 21, 26, 27 for reviews). These findings appear to be in contrast to the existing research on *NR3C1* methylation in healthy adults with a history of childhood adversity, as well as the findings of the present study, which might be attributable to methodological differences in the investigated CpG sites. Tyrka et al. (33) investigated 13 different CpGs from whole blood samples encompassing exon 1_F. The authors reported higher methylation after maltreatment for men and women, but only in one of the investigated CpG sites, which is known to represent a gene-regulatory site (72). Shields et al. (32) reported higher methylation in *NR3C1* in women who had suffered childhood abuse compared to non-abused women. The samples in their study were analyzed from whole blood samples, and the CpG island shore located downstream of the proximal promoter region in *NR3C1* was targeted. The most recent study, by Alexander et al. (34), investigated whole blood samples in childhood trauma survivors from the same 39 CpGs in exon 1_F as in our study. None of the CpGs was directly associated with early life adversity. However, methylation in CpG_{12} (in line with CpG_{12} in our study) moderated the association between childhood adversity and the cortisol response to a psychosocial stress test. Individuals with higher CpG methylation showed a higher cortisol response than those with low methylation. This effect was not visible in participants without childhood trauma, independent of CpG_{12} methylation. In our study, mean % methylation levels across all 39 CpGs within exon 1_F were investigated in line with Palma-Gudiel et al. (21). Overall methylation across the single CpGs was not associated with childhood adversity. Correlation analyses between single CpGs and the maltreatment score did reveal some significant associations (see **Supplementary Table 2**), which need further investigation possibly in a larger sample size. Higher overall methylation was, moreover, related to higher basal cortisol levels and a less favorable E2/C ratio, although these associations did not reach statistical significance when adjusting for control variables. It can only be suspected that these markers might be indicators of low-grade HPA axis dysfunction. Stress induction using the DEX/CRH test or the Trier Social Stress Test would have provided further insights into whether *NR3C1* promoter methylation was associated with alterations in HPA axis functioning in our healthy sample. Additionally, it needs to be considered that only one of the previous studies also exclusively investigated women (32), while the others included equal numbers of men and women (33, 34). Sex differences should be taken into account when investigating NR3C1 methylation and its effect on HPA axis functioning. Additional explanations such as genetic polymorphisms of genes of interest (31), or individual differences in enzymatic activity of methlytransferase, acting on CpG substrates (73) might explain the discrepant findings among studies in healthy adults.

Our findings point in the direction of a dose-dependent relationship between childhood adversity and methylation in the *ERα* shore in women. Animal research has already revealed that early social experience can affect brain, behavior, and stress reactivities through its effect on *ERα* methylation and expression (48–50). Women are generally at a higher risk of stress-related disorders than men (8, 9), and a history of childhood adversity may potentiate this effect. Our study proposes DNA methylation in the *ERα* shore as one possible mechanism linking childhood adversity and risk of psychopathology in women. Notably, we found the association between early life adversity and the overall *ERα* shore methylation as well as for some single CpGs (see **Supplementary Table 3**) in a non-clinical sample of healthy adult women. It may be speculated that this effect could be even stronger in clinical populations and pathologies such as depression, which is thought to be linked to both estrogen actions and early life adversity (3, 74, 75).

The major strength of our study is the strict inclusion of healthy women. With this approach, we were able to rule out the effect of current psychiatric disorders on methylation and therefore investigate a "clean" sample. Only 11 participants had experienced any lifetime psychiatric disorders (mainly depression or eating disorders), and these participants did not differ from the rest of the sample either in terms of methylation or adversity (results presented in **Supplementary Table 1**).

Nevertheless, there are some methodological limitations which need to be considered when interpreting the findings of our study. Early life adversity was captured with the Childhood Trauma Questionnaire. Despite being one of the most widely used questionnaires in this field, the CTQ does not consider other early life stressors such as early parental death, or prenatal stressors such as mothers' psychopathology, which could have had an additional effect on the epigenetic mark (26). Moreover, the experience of early life adversity was assessed through retrospective self-report, which might have been biased by social desirability. Previous studies in humans mainly assessed methylation from peripheral whole blood samples, although cell composition might pose a potential confounder and methylation

profiles from peripheral cells might not adequately represent the brain state (76). From their review of the animal and human literature, Turecki and Meaney conclude that strong stressors might lead to an adaptation of the epigenome in the brain and even in peripheral cells. As such, easily accessible tissue samples from peripheral blood can be considered as a valid method to assess methylation profiles (26). We used the DBS technique, which is a relatively new sampling method in the context of methylation analyses. The latest publications are promising and suggest that this handy and simple technique is reliable and valid in the assessment of methylation marks (reviewed in 54, 63). As mentioned above, cell composition can be a potential confounder, which was not controlled for in the present analyses and therefore needs to be considered as a limitation. We excluded some biomarker values, because we considered the levels as implausibly high. Although we based these decisions on criteria such as age or menopausal stage and compared levels with findings from previous studies, the exclusion of these values still has to be considered as a possible limitation. Finally, we investigated basal profiles of steroid hormone levels. As discussed above, markers of stress reactivity might provide additional valuable insights into the mechanisms underlying disease and resilience.

To conclude, a large number of high-quality studies suggest a link between early life adversity and the risk of psychopathology later in life. The precise underlying biological mechanisms are, however, still a subject of study. For the purpose of comparability, future studies, using sex-specific analyses, are encouraged to consider the mediating role of single CpG and overall *NR3C1* promoter and *ERα* shore methylation in the association of early life adversity with both basal hormone secretion and endocrine stress reactivity in men and women. The finding regarding *ERα* shore and early life adversity needs to be replicated in further studies employing larger sample sizes, women at diverse developmental stages, and clear indicators of the source and strength of adversity. In particular, studies in children or adolescent girls could be crucial, as this would enable the effects of further lifetime stressors on the epigenetic mark to be ruled out or controlled for. Only in this way can other pathways of action, which might provide a differential or additional explanation for increased *ERα* shore methylation, be ruled out. If replicated, this mechanism of action might provide more insights into specific pathways linking early life adversity and disease, especially in women. In conclusion, rather than one single biological mechanism, a complex interplay of characteristics of exposure, sex, and biological resources in the form of genetics and epigenetic marks, with subsequent consequences for stress adaptation, seems to meditate the effect of early life adversity on risk or resilience.

DATA AVAILABILITY STATEMENT

The datasets for this manuscript are not publicly available due to data privacy regulations. Requests should be directed to s.fiacco@psychologie.uzh.ch.

ETHICS STATEMENT

The study was conducted in accordance with the recommendations of the Cantonal Ethics Committee (KEK) Zurich, which classified the protocol as uncritical. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

UE, SF, EG, LM, and LS contributed to the conception and design of the study. SF, LM, and LS collected data. EG performed methylation analyses. SF performed the statistical analyses; all authors contributed to data interpretation. SF wrote the first draft of the manuscript. EG wrote the methods sections on methylation. All authors contributed to manuscript revision and have read and approved the submitted version.

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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.2019.00777/full#supplementary-material

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REVIEW
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Epigenetic Modifications in Stress Response Genes Associated With Childhood Trauma

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Adverse childhood experiences (ACEs) may be referred to by other terms (e.g., early life adversity or stress and childhood trauma) and have a lifelong impact on mental and physical health. For example, childhood trauma has been associated with posttraumatic stress disorder (PTSD), anxiety, depression, bipolar disorder, diabetes, and cardiovascular disease. The heritability of ACE-related phenotypes such as PTSD, depression, and resilience is low to moderate, and, moreover, is very variable for a given phenotype, which implies that gene by environment interactions (such as through epigenetic modifications) may be involved in the onset of these phenotypes. Currently, there is increasing interest in the investigation of epigenetic contributions to ACE-induced differential health outcomes. Although there are a number of studies in this field, there are still research gaps. In this review, the basic concepts of epigenetic modifications (such as methylation) and the function of the hypothalamic-pituitary-adrenal (HPA) axis in the stress response are outlined. Examples of specific genes undergoing methylation in association with ACE-induced differential health outcomes are provided. Limitations in this field, e.g., uncertain clinical diagnosis, conceptual inconsistencies, and technical drawbacks, are reviewed, with suggestions for advances using new technologies and novel research directions. We thereby provide a platform on which the field of ACE-induced phenotypes in mental health may build.

Keywords: childhood trauma, stress disorders, mental health, the hypothalamic-pituitary-adrenal axis (HPA), epigenetic association studies

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ADVERSE CHILDHOOD EXPERIENCES/CHILDHOOD TRAUMA

Stressful or traumatic events experienced in childhood or adolescence can be driven by a broad range of life events, including but not limited to physical injury, natural disaster, bullying, and childhood maltreatment (1). They are referred to by many terms, including early life adversity, early life stress, early life trauma, and adverse childhood experiences (ACEs) (2). It is reported that the worldwide average trauma exposure rate is 69.7% for children and adults (3). In the United States, around 60% of adults reported that they had experienced at least one type of ACE (2).

ACEs/childhood trauma are associated with negative health outcomes, both mentally and physically (4). Individuals exposed to multiple types of childhood trauma show an increased risk of early mortality, which decreases their lifespan up to 20 years (5). Physically, childhood trauma has been associated with increased risk of cardiovascular disease (6), autoimmune disease (7), gastrointestinal symptoms (8), poor dental health (9), obesity, and type 2 diabetes (10). Psychologically, childhood trauma is regarded as one of the major risk factors for psychopathology. Childhood trauma has been associated with many mental disorders (11). Specifically, childhood trauma has been linked to posttraumatic stress disorder (PTSD) (12), insomnia (13), anxiety (14), depression (15, 16), bipolar disorder (17, 18), maladaptive daydreaming (MD) (19), hallucinations (20), borderline personality disorder (21), disruptive behavior (22), risky behaviors (23, 24), substance abuse (25, 26), antisocial behavior (27), and eating disorders (28, 29).

Childhood trauma impacts children to different extents. Some people are more vulnerable, whereas, others show the characteristic of "resilience," with the ability to "bounce back" even after adversity (30). Multiple factors, e.g., genetic, epigenetic, and environmental factors, and their interactions contribute to the differential health outcomes induced by childhood trauma. According to a neural diathesis-stress model, genetic predisposition and environmental factors contribute synergistically to the development of mental disorders. The magnitude of the heritability of a phenotype is one way of estimating the relative magnitude of the genetic contribution. In the case of ACE-associated psychiatric disorders such as PTSD, the heritability is in fact low to moderate (31). Similarly, the heritability of resilience is low to moderate, varying in research reports from 25% to 60% (32–34). These heritability values suggest that there may be other mechanisms contributing to these phenotypes, such as gene by gene interaction and gene by environment interactions, and epigenetic mechanisms. Consequently, it might well be productive to explore genetic, epigenetic, and environmental interactions in resilience and ACE-associated health outcomes.

THE ASSOCIATIONS BETWEEN GENETIC AND EPIGENETIC AND CHILDHOOD TRAUMA

Epigenetic Modifications

The human genome is made up of DNA, which stands for deoxyribonucleic acid, the genetic code which is a continuous sequence of four "letters" or "bases," A, G, C, T (A = adenine, C = cytosine, G = guanine, T = thymine). This encodes heritable information from parents to offspring. Part of this sequence is "read" during a process known as "transcription." Transcriptional machines, which have a complicated structure and are made up of several protein subunits, are needed to start this process. Following transcription of the locus, the noncoding DNA areas (known as "introns") are spliced out and the regions that are coding proteins/peptides, known as "exons," are converted into mRNA sequences. These mRNAs are then used to build different protein structures from "building blocks" known as amino acids. In the next, "posttranslational," stage, further modifications may occur and influence the function of the protein. In general, gene expression is a complicated dynamic process and controlled by diverse regulators at different levels, such as transcriptional regulation (*cis*: e.g., promoters, *trans*: e.g., DNA-binding proteins), RNA processing (RNA splicing, noncoding RNA, miRNAs, etc.), translational regulation, and posttranslational regulation (acetylation, phosphorylation, and glycation, etc.) (35).

Epigenetic modifications regulate this dynamic process of DNA to protein. Epigenetics, which means "outside conventional genetics" (36), focuses on the regulation of "turning on or off" genes without changing the DNA sequence, but rather the accessibility of regulatory transcription factors to the gene. Epigenetic modifications impact on multiple nuclear processes, such as DNA packaging and chromatin structure, and thus on gene expression, with various directions of effect (which may be conceptualized as "epigenetic readers, writers, and erasers") (37). Such modifications include changes in the spatial positioning of chromosomal territories (38). There are three main types of epigenetic modifications: DNA methylation, histone modifications, and various RNA-mediated processes (39, 40). Epigenetic modifications may be cell-type-specific.

Cytosine methylation (5-methylcytosine, 5-mC) is very common in both eukaryotes and prokaryotes (41). It largely happens at cytosine followed by guanine residues (CpG) sites; less commonly, cytosine may be methylated at CpA, CpT, or CpC sites. A family of enzymes named DNA methyltransferases (DNMTs) regulates DNA methylation through transferring a methyl group to the DNA base cytosine (42). Methylation, which is similar to a protective cover on the DNA, generally suppresses gene expression by physically preventing transcription factor binding (43). It also suppresses gene expression by interacting with other mechanisms, e.g., histone deacetylase (HDACs) complex recruitment. For example, methyl-CpG-binding proteins (MeCP) 2 binds tightly to chromatin in a methylation-dependent way, which induces the formation of the histone deacetylase complex. This complex induces transcriptional suppression by changing chromatin structures (44). However, DNA methylation also enhances gene expression through more complicated mechanisms such as the methylation of CCCTC-binding factor (CTCF) binding sites and/or intronic regions (45–49). Hydroxymethylcytosine (5-hmC) is another form of DNA methylation. It is in fact converted from5-mC through an oxidative reaction, by the ten-eleven translocation methylcytosine dioxygenase (TET) 1 enzyme. Similarly, 5-hmC is able to both activate and suppress gene expression in a bidirectional manner (50). The expression of 5-hmC is highly concentrated in the brain and is dynamic during fetal development (51). It has been reported to play important roles in neuronal function, learning and memory, and stress-mediated responses (52).

As for histone modification, it impacts chromatin structure and DNA packaging (37) [e.g., the acetylation of histones may render DNA more or less accessible to transcription factors, leading to enhanced or reduced transcriptional activity (53)]. It has been estimated that the full length of DNA is more than 500 billion kilometres in the human body (54). Consequently,

DNA needs to be packed tightly into cells. Histones are directly involved in the packaging process. Basically, a core of DNA (around 146 base pairs) wraps around each histone to form a structure known as a "nucleosome." Subsequently, an octamer comprising four different histones (H3, H4, H2A, and H2B) is formed. This is further packed into chromatin fibres and then into chromosomes, a unit now visible under a light microscopes. There are several types of histone modifications, including acetylation, methylation, phosphorylation and ubiquitylation. The specific modification patterns of histones, which are called histone codes, work with the other chromatin associated proteins, change the structure of the local chromatin, and thus impact the process of gene expression, such as transcription, replication and DNA repair. The proper topological structure of chromatin is essential in gene expression and genome maintenance (55).

Lastly, noncoding small RNAs (e.g., miRNAs) are also able to mediate sequence-specific modulation of gene expression by different mechanisms (56). For example, miRNAs bind to their target mRNAs *via* complementary sequences, which induces the cleavage and degradation of the corresponding mRNA (57). More recently, additional epigenetic modifications have been discovered, including for example, RNA methylation (58).

Each cell in the living organism, under normal conditions, essentially shares the same copy of DNA, but eventually develops and differentiates to different cell types under regulatory mechanisms. Epigenetic modifications such as genetic imprinting (59) are necessary for embryogenesis and gametogenesis (60, 61), differentiation, and development. In fact, epigenetic regulation occurs throughout the lifespan and can be induced by random changes (62) or by multiple different environmental factors (63). For example, changes in human epigenome have been associated with processes related to adaptation and evolution (64, 65), and have also been linked to several diseases, such as cancer (66), type 2 diabetes (67), and autoimmune rheumatic disorders, such as systemic lupus erythematosus (SLE) (68). Epigenomic alterations are also associated with pathologies characterized by behavioral or/and cognitive problems, e.g., Alzheimer's disease (69), Rett syndrome (70), Cushing's syndrome (71), depression (72), addiction (73), aggression and antisocial behavior (74), and also with illnesses characterized by childhood trauma exposures, such as mental disorders (75).

Early life is a special period characterized by a high level of plasticity and fast development (76). Thus, the impact of childhood trauma is particularly deleterious, since the developmental trajectory of the brain is affected, with resultant alteration of the circuitry for threat detection, emotional regulation, and the reward system (77).

In this paper, we will focus on the epigenetic modification of DNA methylation, as this has the most data relevant to childhood trauma.

The Associations Between Methylation and Childhood Trauma

Stress and the HPA Axis

Why does childhood trauma impact health outcomes? One mechanism is by the induction of toxic stress. In fact, stress can be classified into "good stress," "bearable stress," and "toxic stress" (78), and has acute, delayed and long-term effects on the body (79). "Good stress" can be coped with by physiological mechanisms, encouraging healthy growth; "bearable stress" states may eventually be turned into homeostasis through successful interventions; whereas, "toxic stress," which is characterized by prolonged or frequent activation and dysregulation of the stress response pathway, induces long-term changes and damage not only to the brain but also to the rest of body (2, 80).

The central biological pathway involved in the response to stress is the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1**). In 1914, Walter B. Cannon put forward the "fight or flight" model, which described the body's response toward stress (81). Around the 1950s, Selye's general adaptation syndrome was put forward: that chronic stress could induce a nonspecific response in the body, such as increased heart rate and blood pressure (82). More recently, more in-depth research has illustrated that alterations in the HPA axis have been associated with the process of dealing with stress, especially toxic stress-induced negative health outcomes (83).

When a threat signal is recognized, the central nervous system (CNS): amygdala (84), hypothalamus (85), and parts of brainstem such as the locus coeruleus, (86–88), which are regarded as the central components of the stress response, will be activated. Neurotransmitters such as glutamate, serotonin (89), and γ-aminobutyric acid (GABA) are involved in this signal transmission. On receipt of the neuronal signal from the amygdala and locus coeruleus, numerous neuropeptides are released from the hypothalamus, including arginine vasopressin (AVP) and stress-induced corticotropin-releasing factor/hormone (CRF/H) (90). The CRF Receptor 1 ($CRFR_1$) on the anterior pituitary is activated, which results in the secretion of adrenocorticotropic hormone (ACTH). AVP works together with CRH to contribute to the ACTH response (91). ACTH acts on receptors in the adrenal cortex, leading to the release of stress-related hormones: glucocorticoids (cortisol) and mineralocorticoids (aldosterone). These stress-related hormones mediate the stress response (92) to induce changes in heart rate, blood pressure, metabolism (93), and immune function (94). Other neuropeptides/neurotrophic factors, such as neuropeptide Y (95), dynorphin (96), and oxytocin as well as brain-derived neurotrophic factor (BDNF), are also involved in the HPA axis and in the orchestration of the response to stress.

On the other hand, in the sympathetic adrenal medullary (SAM) axis, a signal from the hypothalamus activates the adrenal medulla, and then induces the secretion of the catecholamines adrenaline and noradrenaline. Peripheral organs (e.g., heart, liver), glands, and vessels have receptors for these hormones and are in addition regulated by the sympathetic autonomic neurons. Together with the HPA axis as mentioned above, the downstream effects, e.g., increased heart rate and blood pressure, are intended to be biologically adaptive, to enhance the individual's ability to respond to the stressor.

Importantly, cortisol provides negative feedback are the level of the hypothalamus (97) to stop the HPA axis from being excessively activated with consequent deleterious health effects. In addition, within the autonomic nervous system, parasympathetic

neurons balance the activation of the sympathetic system, inducing a "rest and digest" body state. Childhood stress and trauma alter the HPA axis (98) and the long-term dysregulation of the HPA axis induced by childhood stress/trauma has been associated with increased risk of adverse health outcomes. For some of these, the effects of adversity appears to be dose-dependent (99–101).

Hotspot Genes

There is increasing interest in the investigation of epigenetic and environmental interactions in ACE-induced differential health outcomes. In humans, studies have mainly focused on peripheral tissues, such as peripheral blood, buccal cells, or saliva. There are also studies with human postmortem brain tissue. For example, Labonte and colleagues reported that in hippocampal tissues derived from those who had died by suicide, comparing those with and without a history of childhood abuse, there were 362 differentially methylated promoter sites. Among these, 248 sites were hypermethylated and 114 were hypomethylated (102). Similarly, there was a bidirectional regulation of methylation in the cingulate cortex of those with/without childhood trauma who has had depression and died by suicide, with the highest differential methylation occurring in genes that related to myelin (103). In a 2017 systematic review of epigenetic associations with childhood trauma in first episode psychosis patients and healthy individuals, childhood trauma was associated with global hypomethylation in peripheral blood samples (104, 105).

A key limitation of such epigenetic research as described above is nonetheless the tissue specificity of effects, which means that for only very limited sites scan congruent changes across tissues be expected (106, 107). In fact, even with the same sample, e.g., saliva taken at different times from the same individual, the cellular composition (proportion of different cells) may vary, which brings challenges to the analysis of methylation results (108).

Relevant biological systems relevant to the HPA axis are summarized in **Figure 1** with highlights provided below.

FKBP5

The *FKBP5* gene encodes a heat shock protein 90 (HSP90) cochaperone that modifies the sensitivity of steroid receptor hormones, interacting with the glucocorticoid receptor (GR), the progesterone receptor (PR), and the androgen receptor (AR). Together with other chaperone proteins such as Hsp90, FKBP5 inhibits GR function by slowing ligand-receptor complex translocation to the nucleus (109). It has been reported that in the HPA axis, the activation of GR inhibits the expression of CRH and ACTH, thus restraining overactivation of the HPA axis (110). Although GR activation increases the expression of *FKBP5*, the increased binding of *FKBP5* to the GR suppresses GR

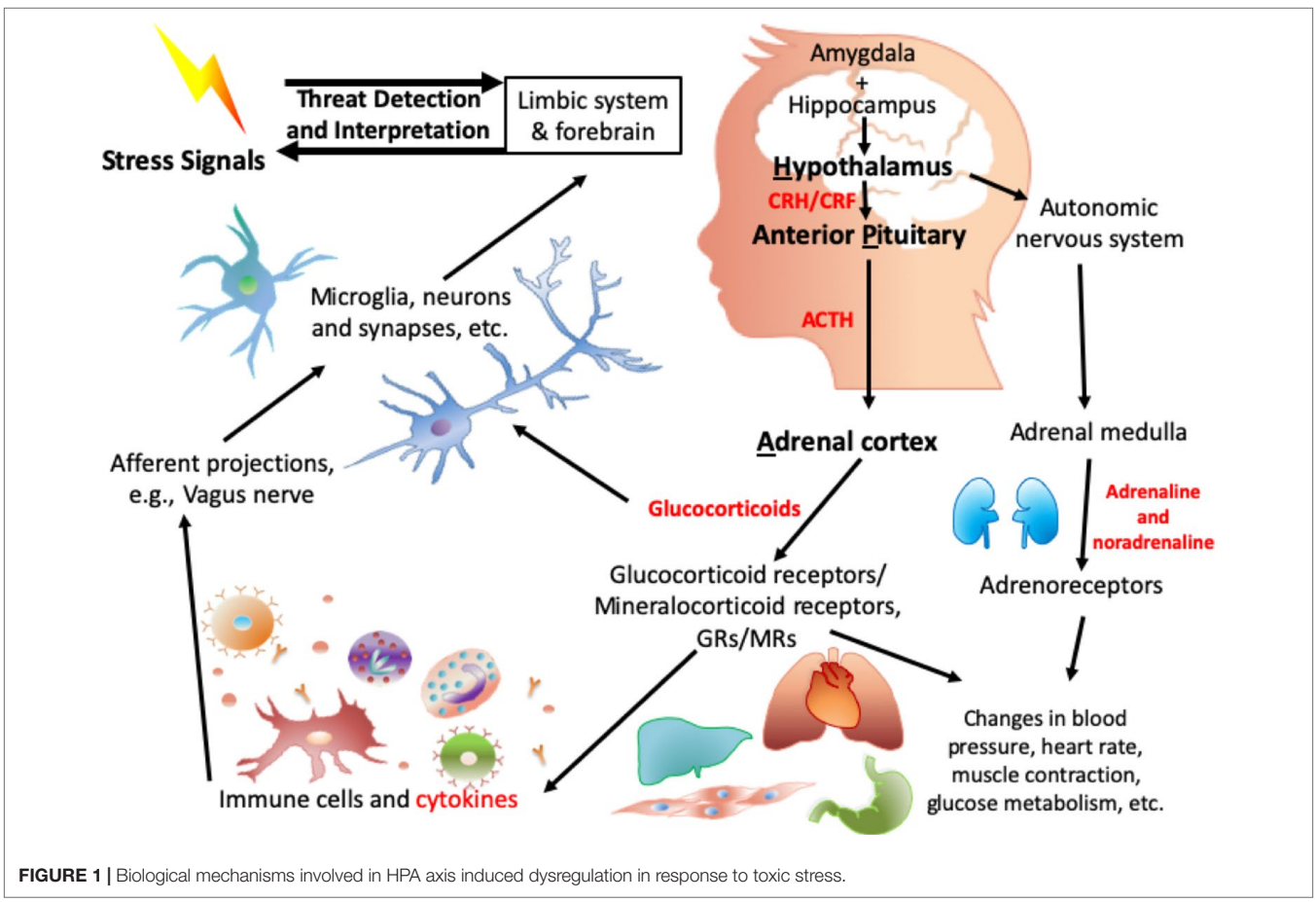

FIGURE 1 | Biological mechanisms involved in HPA axis induced dysregulation in response to toxic stress.

activity in a negative-feedback way. Thus, alterations in *FKBP5* hinders this negative feedback loop and induces "glucocorticoid resistance" (111).

Genetic variants of *FKBP5* impact gene expression. For example, the SNP rs1360780, which has been associated with a change in the three-dimensional structure of the genetic locus, influences the physical contact between RNA polymerase and the transcription start site, as well as the hormone responsive elements (HREs) located in intron 2 (112, 113). Consequently, *FKBP5* genetic variants may induce differential health stress-related outcomes *via* their influence on GR sensitivity (114), the HPA axis, and subsequent regulation of neuronal function and synaptic plasticity (115). Exposure to childhood trauma has been shown to interact with the rs1360780 risk allele (T) to increase risk for a number of psychiatric disorders (115). It has been proposed that rs1360780 risk allele carriers show an increased *FKBP5* mRNA response on exposure to stress (through enhanced binding of the promoter and the intron 2 glucocorticoid response element GRE), along with decreased negative feedback signal to the GR, which induces prolonged cortisol secretion. Enhanced cortisol secretion induces decreased DNA methylation in the intron 7 GRE, which in turn further enhances the activation of *FKBP5* (116). This dual genetic and epigenetic disinhibition may increase FKBP5 levels and induce downstream changes in cellular and circuit function to a level that promotes risk for psychiatric disorders (116). For example, in major depressive disorder (MDD) patients who have been exposed to childhood trauma, the risk allele (T) of rs1360780 has been associated with a lower methylation level of *FKBP5* in peripheral blood cells, and lower methylation of *FKBP5* has been linked to functional as well as to structural alterations in the inferior frontal orbital gyrus (117). This region of the brain belongs to the anterior limbic and paralimbic structures and plays an important role in response inhibition and cognitive function (118). Also, alterations of this area have been associated with symptoms of PTSD induced by childhood sexual abuse (119). Interestingly, it has been found that the *FKBP5* rs3800373 minor allele alters the secondary structure of *FKBP5* mRNA, decreasing the binding of a stress- and pain-associated microRNA, miR-320a. This results in relatively greater *FKBP5* translation, unchecked by miR-320a, increasing glucocorticoid resistance and increasing vulnerability to stress such as posttraumatic pain (120).

MAOA

Other genes that have been associated with the effects of childhood trauma are the *monoamine oxidase (MAO)* gene (located on the X chromosome), encoding the mitochondrial bound isoenzyme MAO A and B, which break down monoamine neurotransmitters such as dopamine, serotonin and noradrenaline (121). This degrading function of MAOA and MAOB is essential for the maintenance of synaptic transmission and thus proper motor control, emotional regulation, and cognitive function (122). There are more data relevant to this review on *MAOA* than on *MAOB*.

In 1993, it was reported that a point mutation in exon eight of the *MAOA* gene (leading to a premature stop codon) contributes to Brunner Syndrome, with prominent aggressive behaviors (123). Polymorphisms in *MAOA* have in fact been associated with antisocial behavior in general (27, 124), as well as with panic disorder (125), restless legs syndrome (126) and attention control (127).

The variable number tandem repeats (VNTRs) in the *MAOA* gene have been associated with differential health outcomes after stressful life events. VNTR may be variously defined, generally referring to short tandem repeats of 20–100 nucleotides. They regulate gene expression and have been associated with human diseases (128) such as spinocerebellar and Friedreich ataxia, fragile X syndrome, Huntington's disease (128), and other psychiatric disorders.

There is a VNTR comprising CCCCTCCCCG (known as the "A repeat") and CTCCTCCCCG (known as the "B repeat") of a 10-base pair unit near the transcription start site (TSS) of *MAOA* that contributes to antisocial behavior after exposure to childhood trauma in females (129). The first six repeats are the same within different individuals, ABABAB; variants occur in at or after the seventh repeat. For example, seven repeats (7R) is ABABABA, and 10 repeats (10R) is ABABABAAAA. The risk allele (comprising 10 repeats) is associated with lower *MAOA* activity. Lower *MAOA* activity, which is associated with higher levels of the relevant neurotransmitters, has been associated with increased risk of conduct disorder and antisocial behavior (130, 131). Another well-studied VNTR in the *MAOA* gene is the 30 base pair (bp) upstream VNTR (u-VNTR) with a repeat sequence of 5'-ACCGG CACCGGCACCAGTACCCGCACCAGT-3' (132). The risk allele is three repeats, which has been associated with a significantly decreased level of *MAOA* expression (133). Similarly, maltreated children with the risk *MAOA* u-VNTR genotype develop conduct disorder, antisocial personality, and violent criminality in adulthood (131).

Moreover, different genetic variants have been associated with differential methylation status of *MAOA* and corresponding phenotypes. For example, nine-repeat (9R) carriers (the lower risk allele) of the 10 bp *MAOA* VNTR show a lower methylation level in the *MAOA* promoter in females (129). In regard to the 30 bp u-VNTR, carriers of the lower-MAOA-activity variants (i.e., the higher risk alleles such as 3.5) had a higher risk of depression with histories of childhood trauma in females compared to those who without trauma histories, and the overall methylation of *MAOA* was reduced in depressed patients (130). Interestingly, the lower-activity MAOA-allele (3.5 repeats) of the *MAOA* u-VNTR has been associated with other epigenetic modifications, such as *NR3C1* hypermethylation after childhood trauma (130).

In monozygotic twin studies, hypomethylation of all *MAOA* CpG sites has been negatively associated with depressive symptoms, but not with childhood trauma; whereas, hypermethylation of the *MAOB* gene shows a nominally significant association with childhood sexual abuse (134).

NR3C1

Another well-studied candidate in the HPA axis is the GR gene: *nuclear receptor subfamily 3 group C member 1 (NR3C1)*. *NR3C1* encodes the GR. The binding of glucocorticoid to the GRs plays important roles in glucose homeostasis (135) and regulates the stress response through both genetic (136) and epigenetic

mechanisms (137). Childhood trauma and early stress alter the methylation status of this gene and its expression.

Research has shown that altered methylation status in this gene has been associated with childhood trauma, especially the CpG sites located in the noncoding first exons of *NR3C1* (138). In a rat model, pups who received less early nurturing behaviors (low licking and grooming (LG) and arched-back nursing (ABN) from the mother rat) presented significantly higher levels of methylation in the exon 1_7 GR promoter nerve growth factor-inducible protein A (NGFI)-A binding site (139). Since NGFI binding decreases GR expression, this alteration is thought to be associated with impaired regulation of the HPA axis (140, 141).

In humans, it has been shown that experiencing childhood trauma increases methylation of *NR3C1* (142). In Melas and colleagues' study, one specific type of childhood trauma (parental death) was associated with hypermethylation of the *NR3C1* CpGs close to the NGFI-A binding site, at, in association with the L-allele (3.5 repeats) of the *MAOA* u-VNTR in salivary DNA samples (130). In postmortem brain tissue (hippocampus) from those who have died by suicide, there was decreased expression of GRs, along with enhanced cytosine methylation of the *NR3C1* promoter in those with a history of childhood trauma. Also, in the same group there was decreased NGF1-A transcription factor binding and NGF1-induced gene transcription (137). Labonte and colleagues also investigated methylation and *NR3C1* expression in postmortem (suicide) brains. In the hippocampus, total GR expression, and the 1_B,1_C, 1_H GR RNA variant levels decreased with history of childhood trauma. Site-specific methylation showed that the methylation of variants 1_B and 1_C was negatively associated not only with their expression but also with total GR mRNA level. Variant 1_H was associated with effects in the opposite direction (143). Other tissues, such as cord blood, peripheral blood, buccal epithelial cells and placental tissues were also tested, and the majority of them showed similar results in regard to enhanced methylation of *NR3C1* in association with early life adversity (138, 144).

Stressful life events occurring slightly later in life, such as in adolescence, are associated with a further independent increase in methylation of *NR3C1* (142). Interestingly, the effects of methylation within the *NR3C1* promoter can be sex-specific. Vukojevic et al. showed that enhanced methylation of *NR3C1* promoter at the NGFI-A binding site has been associated with less intrusive memory, and thus decreased risk of PTSD among survivors of the Rwandan genocide, but only in males (145).

In a recent study in mice, hemizygosity of a deletion of *nr3c1* (*nr3c1*$^{-/+}$ heterozygote) has been associated with changes in DNA methylation in fetal placenta, and these changes have been associated with methylation changes in the adult prefrontal cortex, as well as with increased anxiety-like behaviors in the same animals (146). In addition, hydroxymethylation modifications of n*r3c1* have been suggested to be involved in the stress response pathway. Li and colleagues reported that acute stress induces accumulation of 5-hmC in the *nr3c1* 3' untranslated region (UTR) in the mouse hippocampus (147). Further investigation of molecular mechanisms involving 5-hmC and childhood trauma in not only *NR3C1* but also in other genes could be productive.

HTRs and SLC6A4

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. It can be found in the gastrointestinal (GI) tract, blood platelets, and the CNS (148). In the CNS, serotonergic neurons are widely distributed in the brain, especially the limbic system (149). Serotonin contributes to brain development (149) and to maintenance of normal brain function. It promotes neural and glial cell growth, differentiation, survival and synapse formation (150). Alterations in the serotonin system have been associated with structural and functional changes in the brain (149). Stress induces brain serotonin turnover (151, 152). Excessively raised serotonin is associated with neurotoxicity (153). Stress-induced serotonin turnover has been associated with conditions such as impulsive violence (154), depressive symptoms (155), and substance dependence (156).

The *HTR* genes encode serotonin receptors, which are widely distributed in the CNSs including the prefrontal, parietal, and somatosensory cortices as well as the olfactory tubercle. Variants in these gene have been associated with differential treatment responses and with various psychiatric disorders, such as panic disorder (157), impulsive disorder (158), PTSD (159), and eating disorder (160). In children, *HTR2A* variants are related to differential risk of being hyperactive (161) with harsh parenting styles (162, 163) or after experiencing childhood abuse (164). It has been reported that early life adversity alters *HTR2A* methylation, and the effects were allele-specific. Contextual stress experienced in childhood induces enhanced *HTR2A* methylation at site-1420, in those of A/A genotype at rs6311- (-1438 A/G). Moreover, enhanced methylation of *HTR2A* at site-1420 was negatively associated with PTSD and depression; whereas, those of G/G genotype presented decreased methylation (165). Notably, hypermethylation of site-1420 has also been found in schizophrenia and bipolar patients (166). In the *serotonin 3A receptor (HTR3A)* gene, the mother's life-time exposure to interpersonal violence is associated with altered methylation in the *HTR3A* gene, which has been associated with their children's secure base distortion (167). In addition to the *HTR2A* and *HTR3A*, there are several other serotoninergic genes that undergo epigenetic modifications and have been associated with childhood trauma, such as that encoding the serotonin transporter.

These serotonin transporter (responsible for serotonin reuptake) encoded by *SLC6A4* (also known as the *5-HTT* gene) is in fact a frequently studied candidate in psychiatric genetics and epigenetics. Higher methylation of *SLC6A4* has been associated with childhood trauma, and with worse clinical presentation in MDD (168). In women, there is a significant association between sexual abuse and *SLC6A4* hypermethylation, which has been linked to antisocial behavior (74). In newborn babies whose mothers have depressive symptoms in the second trimester, methylation at *SLC6A4* promoter CpG sites is lower in both the newborns and in the mothers compared to controls (169). Methylation also mediates allele-specific cortisol response patterns of the *5-HTT linked polymorphic region* (*5-HTTLPR*) (rs25531) (170). The *5-HTTLPR*, consisting of a 20 to 23 bp GC-rich VNTR repeated 14 times in the short allele (S) and

16 times in the long allele (L), is located 1 kb upstream of the *SLC6A4* gene. The short variant is associated with reduced *5-HTT* expression (171). The S/S *HTTLPR* genotype has been associated with increased risk of depression and suicide attempts after stressful events (172), as well as with adult depression after childhood trauma (173). In Alexander and colleagues' study, it was showed that those of S/S genotype with lower methylation level exhibited higher cortisol response; while the association of the *5-HTTLPR* short allele with enhanced cortisol response disappeared at a higher *SLC6A4* methylation level (170).

BNDF

BDNF has been investigated not only for association with childhood trauma, but also for association with mental health outcomes such as psychotic or depressive symptoms (174–176). *BDNF* encodes a neurotrophin, which promotes the growth, differentiation and survival of neurons. BDNF is also involved in neuroplasticity. Structural brain changes are seen after trauma and BDNF is hypothesized to be involved in these (177). For example, early isolation (one type of ACEs) causes decreased BDNF levels in the amygdala and infralimbic cortex; however, the combination of resocializing and the antidepressant fluoxetine reverses the reduction of *bdnf* in rodents (178). In a rat model, early stress (being abused by caretakers) induces enhanced *BDNF* methylation and decreased *bdnf* gene expression in the prefrontal cortex in exon 9 and 14, which includes the transcription start site (TSS) and cyclic adenosine monophosphate (cAMP) response element and the enhanced methylation persists until adulthood (179). In rodents, the *bdnf* gene contains 9 noncoding exons, each of which can be linked to the protein coding exon IX (9) after splicing, and transcription can be initiated before the protein coding exon in the intronic area. Exon-specific transcription is tissue-specific. Importantly, methylation-induced altered gene expression of BDNF is also cell-type specific (180, 181).

In humans, childhood trauma has been associated with decreased serum levels of BDNF (182). Also, childhood maltreatment induces alterations in *BDNF* promoter methylation (75). It has been shown that a lower maternal care condition is associated with higher *BDNF* DNA methylation levels (183). Furthermore, differential *BDNF* methylation has been associated with structural brain changes. For example, socioeconomic disadvantage has been negatively associated with *BDNF* DNA methylation, specifically at the exon IV promoter site, and this lower level of *BDNF* methylation has been linked to greater thickness of the lateral orbitofrontal cortex (lOFC), medial frontal cortex and decreased thickness of the bilateral lOFC in adolescence (age 12–13) (184). These brain areas are relevant to decision-making, emotion, and memory processing.

In addition, BDNF works synergistically with other genes after childhood trauma, such as the 5-HTTLPR (182), noradrenaline/norepinephrine transporter (NET) and corticotropin releasing hormone receptor 1 (CRHR1) genes (185), as well as tryptophan hydroxylase (TPH) 2 (186). In fact, the BDNF receptor TrkB and GRs, as well as mineralocorticoid receptors, are coexpressed in hippocampal neurons. Additionally, as mentioned above, BDNF directly regulates the HPA axis. The administration of BDNF in vivo induces increased CRH level and reduction of BDNF or its receptor normalizes the CRH level and thus, the HPA axis. This cross-talk between BDNF and CRH may be at least partly mediated by the CREB and MAPK pathway and is involved in the enhancement of fear memory under stress (187).

Other Candidate Genes

There are other candidate genes with at least some data in childhood trauma and epigenetic alterations, such as *COMT*, *IL-6* (188), and *OXTR* (189).

The catechol-*O*-methyltransferaseenzyme encoded by the *COMT* gene on chromosome 22q11.2 (190), is involved in the metabolism of catecholamines including the neurotransmitters dopamine, adrenaline, and noradrenaline, in reactions that involve the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to a hydroxyl group (191). There appear to be epistatic effects between *COMT* and *NR3C1* on working memory (192). In addition, methylation of the *COMT* promoter has been associated with a change in prefrontal cortical connectivity in schizophrenia (193), as well as in depression (194).

Interleukin 6 (IL-6) encodes the IL-6 protein, which is a proinflammatory cytokine. Alterations in IL-6 have been associated with psychiatric disorders, such as depression (195). In addition, patients with schizophrenia and a history of childhood trauma have a pro-inflammatory phenotype (196). Inflammatory factors can in fact be regarded as mediators that connect the environmental stimulus of childhood trauma with clinical symptoms. Changes in the *IL-6* methylation has been associated with childhood trauma related phenotypes. In African American men, there was an association with decreased methylation of *IL-6* and enhanced IL-6 protein level after childhood trauma (197). Importantly, altered expression of *IL-6* can be associated with other genetic variants that are involved in neural pathways. For example, women who carry two short alleles of the *5-HTTLPR* present a higher IL-6/IL-10 ratio when dealing with stress (198).

Oxytocin is a neuropeptide hormone facilitating labor and breastfeeding in mammals. In the brain, oxytocin receptors (OXTRs) are expressed mainly in the central nucleus of the amygdala (cAmyg) and the ventromedial nucleus of the hypothalamus (VMH) (199). The cAmyg regulates the fear response (200) while the VMH regulates a range of behaviors including female sex behaviors (201). Oxytocin and its receptor are involved in the regulation of attachment, social behavior and the stress response (202). In a recent study, there was hypermethylation at *OXTR* CpG sites in children who had experienced childhood trauma, and hypermethylation has been associated with decreased grey matter volumes in the orbitofrontal cortex (OFC), which may be related to insecure attachment styles (189).

Complicated Interactions/Cross-Talk

Research has shown that altered methylation has been associated with childhood trauma-induced phenotypes. Several candidate genes (*FKBP5, MAOA, NR3C1, HTR and SLC6A4, BDNF*) have been discussed in this review. However, the actual regulatory network and mechanisms are more complicated.

Firstly, multiple functional pathways or circuits are involved in processes relevant to stress, including both the reward and the fear

circuits, emotional regulation and executive cognitive function. Secondly, in the HPA axis, molecules and their receptors interact and cross talk with each other. Thirdly, there are potential gene by gene, gene by environment, gene by epigenetic modification, and even epigenetic by epigenetic modifications interactions. All these components influence stress-related phenotypes.

For example, the reward pathway/circuit in the brain has been associated with trait optimism, which has been associated with resilience after stress (203).There are two main reward pathways in the brain: the mesocortical dopamine pathway and the mesolimibic dopamine pathway. Glutamatergic and GABAergic connections are also involved in the reward circuit (204). Similarly, glutamatergic and noradrenergic neuronal signalling (203) and dopaminergic connections participate in neuronal regulation in the fear circuit. In addition, adrenergic receptors (205) and GRs (206) are also involved in fear conditioning. The serotonergic and noradrenergic systems have an established role in mood regulation, while the former is involved in motivation as well, with both anxiogenic and anxiolytic effects (207). Dopamine is relevant to mood regulation too. Enzymes regulating these pathways, such as COMT, MAOA and MAOB, regulate these phenotypes.

At the molecular level, there are different levels of cross-talk. For example, the dopamine D_1 receptor interacts with glutamate-mediated excitatory neurotransmission through protein-protein interactions (208). In addition, serotonin signalling, has been reported to interact with cannabinoid receptors (209). Acting as retrograde synaptic messengers (210), the endogenous cannabinoids, such as anandamide, sleep-inducing substance oleamide (211) and palmitoylethanolamide (212), regulate numerous biological processes such as neuronal migration (213), learning, memory (214), pain processing (215), motility (216), and emotional-and reward-related processing (217–219).Further, both serotonin and endocannabinoids are involved in stress-related phenotypes, such as anxiety (212). In addition, serotonin is also involved in the regulation of GRs, such as in primary hippocampal cell cultures (220) and in the rat brain (221). At the genetic level, it has been reported that different genotypes of the *5-HTT* gene has been associated with the altered GRs' mRNA level under conditions of childhood adversity (222). A variant in *MAOA* gene is associated with differential *NR3C1* methylation (130). For *BDNF*, its expression level responds to stress-related HPA axis activation. Moreover, there is a feedback loop whereby directly regulates CRH, and thus, the HPA axis (187). Besides, as mentioned above, multiple other genes, act in concert with *BDNF* (185). These genes further interact with other genetic/epigenetic variants to form a sophisticated molecular and functional network, which has not yet been fully characterized. For example, *TPH2* also interacts with the *adenosine deaminase, RNA specific B1* (*ADARB1)* gene, which affects pre-mRNA splicing. The interaction of these two genes predicts risk of suicide attempts after childhood trauma (223). A given neurotransmitter/neuronal pathway may conduct more than one function, e.g., glutamate signaling has been associated with both activation and inhibition of the HPA axis through inotropic and kainite/group I metabotropic receptors respectively (224). Interestingly, cognitive therapy and cognitive reappraisal decreases amygdala and HPA activation in response to stress (225), suggesting that there is some "flexibility" in stress-related psychiatric phenotypic presentations. Hence, molecular mechanisms of the HPA axis and the stress response pathway more widely are not only highly complex and orchestrated but also require further illumination.

LIMITATIONS AND NEW DIRECTIONS

Limitations

Limitations exist in this field. Even though numerous studies have been done, evidence of associations between epigenetic/epigenomic alterations and differential health outcomes induced by childhood trauma are limited (226). Additionally, there are inconsistencies in the field. For example, the association between childhood trauma and *NR3C1* methylation has not been consistently replicated (138) and likewise the differences in *SLC6A4* methylation between trauma- and nontrauma-impacted groups (104).

The full complements of molecular mechanisms involved in childhood trauma related health outcomes remain to be elucidated (31). As mentioned above, a further complication is the possibility of coordinate regulation of epigenetic processes in more than one gene/pathway. In addition, there may be pleiotropic or polygenic effects. Pleiotropy means that a gene is associated with more than one phenotype (e.g., the association between *disrupted in schizophrenia 1 (DISC)1* mutations and various psychiatric disorders) (227), and polygenic means that one phenotype may be influenced by several genes (e.g., AOB blood type systems). Moreover, metastable epialleles, differential expression of alleles induced by epigenetic modification during early embryonic development have been identified in genetically identical individuals, and these may also induce phenotypic changes (228). Additionally, study heterogeneities may have limited the conclusions possible in this data synthesis.

Phenotypic Heterogeneity

Between study heterogeneity includes the investigation of different types of childhood trauma. Research has shown that different types of trauma stimulate different brain areas (77). Although psychological trauma might induce similar biological responses to physical trauma (229), the affected brain areas are different: physical stressors mainly impact the brainstem and hypothalamus (230); whereas, psychological stressors mainly impact regions that regulate emotion, learning, memory and decision making, e.g., the hippocampus, the amygdala and the prefrontal cortex (231, 232). Moreover, long-term stress and acute stress have different effects on the brain. Trauma timing, and frequency also impacts differential health outcomes owing to neurodevelopmental stages (233). However, the exact timing as well as the frequency are difficult to reliably record, since the most common type assessment for childhood maltreatment is retrospective self-report, which may map relatively poorly on to prospective assessments (234).

In addition, phenotypic measurement and diagnosis for children who experience childhood trauma may be ambiguous. In diagnosis, children exposed to childhood trauma may develop PTSD, but the potential outcomes are not limited to PTSD (235). Even with PTSD, there are arguments about the diagnostic criteria in DSM-5 (e.g., lack of connection between exposure to stressor and some specific symptoms, some very-well-documented symptoms failing to be captured in DSM-5, and lack of extensive field trial data (236)). Consequently, the term 'complex PTSD' has been put forward to describe complicated traumatic outcomes not captured by standard PTSD (237). Importantly, in behavioral measurement, it is necessary to develop appropriate mathematical models and measurements to correctly quantify within- and between-individual variability (238).In behavioral studies, it is hard to define associations between single genetic/epigenetic variants, as behavioral traits are usually controlled by multiple genes (239). In the definition of childhood trauma induced phenotypes, cultural and ethnic differences may bring additional between study heterogeneity (240). There are other factors such as sex/gender differences in response to stress (241, 242), and the use of different tissues (saliva, cord blood, whole blood and peripheral blood) by different researchers (243). The latter brings complexity to data comparisons since the epigenetic signature differs between and within tissues (244).

Crucially, more than one trait contributes to health outcomes after experiencing trauma. The same genetic/epigenetic modification may impact differently on different traits in one individual. For example, 7 repeat (7R) carriers of the *DRD4* exon III VNTR exhibit the highest sensitivity toward parental-induced stress (245); however, they also show a higher level of emotional resilience due to the association between the 7R and specific personality types (246).

Methodological Heterogeity

Although epigenetics is not a novel concept [the first scientific hypothesis of epigenetics was put forward by Malpighi (247) in 1673, with a key milestone of epigenetic development in 1975 by Riggis (248), Holliday and Pugh (249)], and may simply mean inherited altered gene expression states, it may also refer to inter- versus trans-generational effects, where the former refers to transmission across one generation, and the latter to transmission across multiple generations (250). Historically, these terms have been ambiguously defined (247, 251, 252). This has led to misunderstandings as well as to bias in methodologies and interpretations, especially in interdisciplinary research (253). Indeed, inherited epigenetic patterns (254–256) and environmental factors (257, 258) other than childhood trauma (such as heavy metals (259), parenting style, and early trauma such as maternal separation (260)) may all impact the epigenetic pattern and hence childhood trauma-induced differential health outcomes. However, how much these changes are due to these factors, and to what extent, remains, unclear (261).

In regard to methylation, except for CpG methylation, there are some non-CpG methylations, such as CpA, CpT, and CpC. These are expressed in cell types such as pluripotent stem cells, oocytes, neurons, and glial cells. Importantly, these non-CpG methylations are critical in maintaining neuronal function and are thus involved in neurological disorders (262). Kigar and colleagues posited that adenosine methylation could be regarded as an epigenetic marker of mammalian early life stress (263). However, more research is needed in regard to the above non-CpG methylations, and also that of 5-hmC. As for non-coding RNA, and histone acetylation, there are to date few investigations of associations between these and childhood trauma. Furthermore, the various epigenetic mechanisms, such as methylation, histone modification and noncoding RNA, while often studied one by one, may cooccur and act in concert.

Research has shown that the effects of trauma can be intergenerationally passed on through epigenetic mechanisms, such as methylation (264). Specifically, childhood trauma has been associated with alteration in methylation patterns in human sperm, which may induce intergenerational effects. Further such analyses in larger samples are required (265). Importantly, in addition to epigenetic modifications, other factors, such as epimutations (an mutation occur at the epigenetic level), fetal reprogramming (266), and even gut microbiome transfer (267) may induce intergenerational phenotypic changes. It is challenging and costly to investigate/exclude all of these factors in one human study.

Sex/Gender Differences

Sex/gender differences exist in this research field. In stress-related psychiatric disorders, there are sex-associated differences in incidence, symptoms and treatment response (268). For example, in PTSD, the life time prevalence in females (10-12%) is 2-3 times higher than that in males (5-6%) (269). Similarly, depression is more common in females than males (268). Interestingly, both sex- and gender-related concepts contribute to these differences (270).

There are multiple reasons that may explain these phenomena, such as differential traumatic exposures, cognitive factors, coping strategies and biological factors between different sexes. There are also fundamental sex-dependent brain differences between males and females, e.g., the size of vasopressin (AVP) neurons (271). Moreover, when dealing with stress, males and females present different sex-specific cortico-striatal and limbic patterns. In the work of Cahill and colleagues (272), men showed greater activation of the right amygdala; whereas, women showed greater activation of the left amygdala when facing stress (272). In addition, brain connectivity in response to stress also differs by sex: e.g., there was greater connectivity between the anterior and dorsal anterior insula, as well as between the anterior and dorsal anterior mid-cingulate in males than females after stress (273, 274).Similarly, Helpman and colleagues showed that males present overactivation and increased connectivity of salience hubs (including anterior insula (AI) and dorsal anterior cingulate cortex (dACC)); whereas, females show an overactive and possibly enlarged amygdala. In addition, males lose more grey matter after stress in limbic system structures (prefrontal

cortex, amygdala and the hippocampus (275). These differences contribute to differential fear processing, emotional regulation and decision-making. Moreover, males and females cope with stress differently. For example, when facing traumatic stress, females tend to be more emotion-focused and to use more palliative coping skills than males. Also, females tend to seek social support more and benefit more from psychotherapies (269). Differential stress-related phenotypes between males and females are also related to the gonadal hormones, which play important roles in the establishment, activation and regulation of the HPA axis (276). In animal models, both female rats and mice exhibit more robust responses of the HPA axis (such as a higher level of ACTH), owing to circulating estradiol. In rats, progesterone and estrogen have been shown to directly impact the stress response in females (277). Epigenetic modifications are also involved in gonadal hormone setting up and maintenance of sex differences in the brain, even before puberty (278). In rodents, it has been shown that females have significantly higher level of methylation in the estrogen receptor-alpha (ER-α) promoter than estradiol treated females or males (279). Note that, early exposure to estradiol induces masculinization/defeminisation (280, 281). Interestingly, these sex-dependent epigenetic changes are dynamic across the lifespan (279).

Current studies in regard to epigenetics and sex-dependent phenotypes mainly focus on steroid hormones and targets related to the HPA axis, such as *NR3C1*, and majority of them are association studies, e.g.,the enhanced methylation of *NR3C1*and PTSD risk (145). There are also neurotransmitter specific effects in sex differences. For example, in a study by Oswald and colleagues, the availability of the dopamine D_2 receptor (D2R) has been associated with childhood trauma and pleasant drug (amphetamine) effects. In males, there was a positive association between childhood trauma and pleasant drug effects but not in females (282), which suggests that there may be by sex differences in the reward pathway after childhood trauma (283). Autonomic systems are also different between males and females (284), which may also contribute to sex differences in stress-induced phenotypes. Groleau and colleagues reported higher methylation of the *DRD2* promoter in women with an eating disorder and a history of childhood trauma versus those without such a history (285). Comparison studies between both males and females are limited, probably owing to the different prevalence within different sexes; in some studies with both females and males, the sample sizes were too small to have enough power; the comparison study between the differences of self-identified gender and biological sex, which may provide us the biological and psychological effects about sex-dependent stress responses, are limited; in addition, current studies are mainly focused on the candidate genes that are related to steroid hormones, and they are mainly association studies, which can't provide the information about the causality. Research about more in-depth molecular mechanisms between different sexes, and their interactions with other genetic, epigenetic, as well as environmental factors is limited. Thus, the epigenetic contribution to sex-dependent stress-related phenotypes is still filed for research exploration.

By sex and gender differences are still relatively new areas of research, and hence replications are required and interactions between the above components remain to be explored (285–288).

Technical Limitations

Interestingly, it has been reported that epigenetic patterns and phenotypic changes can be induced by a single genetic variant, combined with random epimutation (289). Hence, it has been recommended that when investigating epimutations and phenotypic changes, the DNA sequence, replication, GC%, and the topological structure of chromosomal bands, especially in unstable genomic areas, should be first analyzed (290) - in an integrated combined "omic" approach. Chromosomal banding was first used with light microscopy and divides chromosomes into regions visible at that level of magnification. These regions include G bands, which have a lower number of genes and lower gene expression level, which replicate late in the cell cycle, and R bands, which have a higher gene number, GC content and expression levels (291). Alterations in the topological structure of chromosomal bands have been associated with changes in gene expression and thus with phenotypes (292–294).

In epigenome-wide association studies (EWAS), although these provide the opportunity to investigate epigenetic variants (methylation, noncoding RNA and histone modification) on a genome-wide level, which could assist with identification of disorder-related markers in different populations (295), the individual CpG sites detected by array methods are limited (296). Genome-wide sequencing approaches can be helpful, but DNA methylation sequencing at a depth to reliably detect the small changes often observed in mixed tissues in human studies is very costly. Targeted assays with high sensitivity covering functionally relevant regions could be an interesting complement here (297). Nonetheless, issues such as cost, speed of delivery, errors of variant annotation, logical and methodological issues (e.g., the appropriate selection of the cohort, population stratification and statistical approaches) remain in human genomic and epigenomic studies (298, 299). Consequently, multiple validations *via* more than one method might bring more reliability.

New Directions

New technologies and strategies have emerged in this field. For example, the nanopore sequencing framework, able to distinguish five types of methylation variants with high-throughput (300). The usage of this technology reduces sample preparation processes and increases the detection speed (300). In addition, nanopore sequencing is able to detect 5-hmC (301), which is not adequately covered by traditional array/bisulfite sequencing methods. We suggest a more in-depth investigation of molecular mechanisms including 5-hmC in relation to childhood trauma related effects.

In living cells, fluorescence recovery after photobleaching (FRAP) has been reported to be able to detect histone mobility (302), which permits real-time investigation of dynamic histone modification. In regard to chromatin structure, Stevens and

colleagues reported that the combination of chromosome conformation capture (3C) and tagged fluorescent imaging was able to detect the folding of a genomic sequence <100bp in a single cell (303). This provides the opportunity to investigate how epigenetic modifications dynamically and spatially mold chromosomes and thus, cellular function and related phenotypes in animal models *in vivo*.

In addition, the CRISPR-CAS9 system can be used to study targeted genetic/epigenetic variant-induced phenotypic changes in animal models. In fact, usage of a modified CRISPR-cas9 system has been expanded beyond genome editing, to RNA targeting, chromatin topology, chromatin imaging, and developmental trajectories as well as to lineage tracing (188, 304). Since the effects of childhood trauma are neurodevelopmental stage-sensitive, a tracing-based technique may provide us with information about when sensitive periods toward different stress are, and how stress impacts on neuronal differentiation (305). The CRISPR-cas9 system can also be used as an effective tool to edit the epigenome (306). Liao and colleagues reported that the endogenous gene was activated *via* trans-epigenetic remodelling by using a CRISPR-cas9 system, and phenotypic changes were observed in acute kidney injury, type 1 diabetes and Duchenne muscular dystrophy rodent models (307). Thus, epigenome editing may help us to better understand the molecular mechanisms in diverse stress-related phenotypes with known targeted sequences. More in-depth molecular insight may also be helpful for improving the definitions and diagnoses of different psychiatric phenotypes.

Given the cell-type specificity of epigenetic changes, achieving single cell-, or at least single cell type-resolution is also an important goal. Single cell sequencing is able distinguish methylated changes in different cell types, and thereby reduce in errors/bias. Using such techniques in combination with sex-dependent stratification, different network mechanisms in males and females may be distinguished. So far, a number of single cell sequencing techniques have in fact been developed to facilitate investigation of methylation (308). For example, single-cell nucleosome, methylation and transcription sequencing (scNMT sequencing), combining epigenome and transcriptome data, are able to detect several "layers" of epigenomic and molecular dynamic coupling processes (309). Psychiatric disorders are more regarded as network dysfunctions (310). As mentioned above, focusing on only one cell type, brain area or neuronal pathway may not be sufficient. Thus, a combination of single cell sequencing and a pathway approach to the analysis of methylation patterns similar to network analysis in genomics (as exemplified by weighted gene coexpression network analysis or WGCNA) could be fruitful in this field.

Furthermore, the assay for transposase-accessible chromatin by sequencing (ATAC-seq) is able to get access to DNA sequences in open chromatin and to produce high quality data with a low background in a high-through output way (311). When being used at the single cell level, ATAC-seq detects DNA regulatory variations, e.g., *trans*-factors, *cis*-elements, which have been associated with induction or suppression of cell-to-cell variability. Such DNA variation data can be combined with chromatin accessibility and thus form a three-dimensional informative "regulome" in the genome (312). The concept of "connectomics" put forward by Fornito and colleagues, may also benefit this field of research (313). "Connectomics" was originally characterized as brain-network topological regulation of neural activities after injury (313). The combination of the different "omic," such as genomic, epigenomic, transcriptomic, and even connectomics studies, may form interesting perspectives about how genetic/epigenetic and their molecular and topological mechanisms impact different cells and brain areas, and thus, stress-related phenotypes. So far, combined "omic" studies such as the combination of GWAS data with enhancer enrichment profiles, RNA sequencing data (RNA-seq) and chromatin status have been utilized (314). The integration of *in vitro* cell culture and multi "omic" analysis in the investigation of human germline epigenome reprogramming has been reported, producing some hints about the origin of neuropsychiatric disorders and transgenerational inheritance (315, 316).

In summary, by using new technologies, "omic" analysis and "big data"-integration of data from different platforms in a system biology approach-bias will be reduced and understanding of molecular mechanisms will be deepened (317). In the future, integration of genomics, epigenomics, transcriptomics, proteomics, metabolomics, regulomics, and connectomics could shed light on both basic biological processes in response to childhood trauma and disorder-related mechanisms, and thereby produce innovations in mental health and addiction health service provision.

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SJ conducted the literature review and drafted the paper for a course (MDGEN605). LP, EB and AC reviewed the manuscript and provided some text and suggested edits. KA reviewed the manuscript, discussed with SJ, provided some text and suggested edits.

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Childhood Adversity Impairs Theory of Mind Abilities in Adult Patients With Major Depressive Disorder

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Background: Patients with major depressive disorder (MDD) have various theory of mind (ToM) impairments which often predict a poor outcome. However, findings on ToM deficits in MDD are inconsistent and suggest the role of moderating factors. Child abuse and neglect are strong predictors of adult MDD and are often associated with a poorer clinical course trajectory.

Objective: Because early-life adversities result in various forms of ToM deficits in clinical and nonclinical samples, our aim was to investigate if they are significant confounding factors of ToM impairments in MDD.

Methods: We investigated 60 mildly or moderately depressed, nonpsychotic adult patients with MDD during an acute episode, and 32 matched healthy controls. The mental state decoding subdomain of ToM was examined with the Reading the Mind in the Eyes Test (RMET). Childhood adversities were assessed with the childhood trauma questionnaire (CTQ) and the early trauma inventory.

Results: There was no difference between the control and MDD groups in RMET performance. However, when we divided the MDD group into two subgroups, one (N = 30) with high and the other (N = 30) with low levels of childhood adversities, a significant difference emerged between the controls and the highly maltreated MDD subgroup in RMET performance. A series of 3 (group) × 3 (valence) mixed-model analyses of covariance (ANCOVAs) revealed that childhood emotional and physical neglect had a significant negative impact on the response accuracy in RMET in general, whereas emotional abuse specifically interfered with the accuracy in the positive and negative valences if it co-occurred with early-life neglect. To test the dose-response relationship between the number of childhood adversities and RMET capacities, we subjected RMET data of the MDD group to multiple hierarchical regressions: the number of childhood adversities was a significant predictor of RMET total scores and RMET scores in the negative valence after controlling for age, sex, years of education, and the severity of current depression.

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Conclusion: Childhood adversities impair ToM capacities in MDD. Exposure to early-life emotional abuse and neglect have a negative impact on the performance in the emotional valences of RMET. Multiple early-life adversities have a dose-dependent association with mental state decoding deficits.

Keywords: adverse childhood experiences, depression, childhood trauma questionnaire, early-life stress, mental state decoding, reading the mind in the eyes test, theory of mind

INTRODUCTION

Patients with major depressive disorder (MDD) have various impairments in social functioning, e.g., withdrawal from social interactions, impaired social competence, negative interpersonal experiences, as well as less enjoyment in social relations (1). Depressed patients can feel self-absorbed, detached from their environment, as well as not knowing how to approach others (2). Typically, the low level of social functioning in patients with depression can be ascribed to social cognitive deficits. Theory of mind (ToM) is one of the essential components of social cognition: the ability to infer the mental states of others by representing their beliefs, intentions, desires, and fears. Hence, ToM is the capacity to attribute mental states (i.e., beliefs, desires) to self and other people, and to understand and predict their behaviors, intentions, and wishes (3). Although the findings are inconsistent, numerous studies detected that patients with MDD have ToM impairments. A recent meta-analysis aggregating 18 clinical studies demonstrated that patients with MDD significantly underperformed healthy controls in different types of ToM tasks (4).

ToM is a multidimensional construct involving several dimensions. Sabbagh (5) identified two components of ToM: (1) the socioperceptual component or mental state decoding: the ability to detect and discriminate cues in the immediate social environment, i.e., the ability to *decode* others' mental states; and (2) the sociocognitive component or mental state reasoning: the ability to infer about social cues, i.e., the ability to *reason* about the mental states of others. Hence, mental state decoding is the initial step of ToM that requires various socioperceptual skills, i.e., adequately identifying and differentiating emotional facial expressions, whereas mental state reasoning abilities encompass sociocognitive capacities, i.e., recognizing conversational failures, as well as interpreting others' emotional states or thoughts.

The Reading the Mind in the Eyes Test (RMET, 6) is a widely accepted ToM test to measure mental state decoding (i.e., the socioperceptual component of ToM). In RMET, participants view a series of black-and-white photographs of the eye region of actors' faces, and they are instructed to judge which of the four adjectives presented simultaneously with the eyes best describe the emotional state of the person in the picture. RMET presents subtle emotional information that embraces a relatively wide range of mental states beyond the basic emotions. Requiring no inferences about cognitive and affective mental contents, as well as no contextual processing, RMET can be regarded as an appropriate task to measure the initial, decoding (or discriminating) ToM processes, predominantly the decoding of subtle facial affective cues. On the basis of the emotional content of the target adjective, RMET photographs have been categorized into three valence groups: neutral (e.g., reflective, thoughtful), negative (e.g., panicked, jealous, hateful), as well as positive (e.g., friendly, playful) (7).

So far, several studies have examined the accuracy of mental state decoding in RMET in clinical and population samples with depressive symptoms as well as in individuals with increased vulnerability to depression (7–13). However, the findings were often controversial, which implies the role of moderating factors such as symptom severity, clinical phase, and feature (including psychotic symptoms, chronicity, as well as acute vs remitted depression). A recent meta-analysis involving seven studies on mental state decoding reported that patients with MDD performed significantly less accurate in RMET (14). Because of the lack of data on other clinical variables and biographic factors, this meta-analysis considered only the effect of psychiatric comorbidities. Nevertheless, the role of early adverse experiences or traumatic childhood events in ToM deficits of depressed patients remained understudied.

However, the negative impact of childhood maltreatment on ToM capacities has been detected in various clinical and nonclinical community samples, e.g., in maltreated youth (15, 16), in a large internet-based, population sample (17), in patients with borderline personality disorder (18, 19) as well as in female patients with post-traumatic stress disorder (PTSD) (20). Moreover, adverse childhood experiences (e.g., physical, emotional, and sexual abuse, neglect, parental loss, and poverty) have long been known to be strong predictors of adult MDD (e.g., 21–23), and early-life adversities were associated with a more severe and persistent course of MDD (24–26). On the other hand, a growing number of data suggests that ToM abnormalities predict relapses and a worse outcome in MDD due to the difficulties in social adjustment (27, 28). Yet, hardly any studies examined the effect of traumatic life events experienced during childhood on mental state decoding abilities in patients with MDD, so far. Merely, a very recent study reported that childhood emotional abuse was associated with poorer response accuracy in RMET in adult patients with MDD, while physical abuse negatively influenced the mental state decoding accuracy of healthy, never-depressed controls (29). In addition, neglect was associated with poorer RMET accuracy across both groups. Although this groundbreaking study focused on the effect of the different forms of childhood adversities, it did not consider the general effect of early-life stress and the effect of multiple adversities on RMET accuracy.

On the basis of the literature, we designed a study in which the mental state decoding component of ToM was assessed in a relatively homogeneous group of MDD patients. We examined the mental state decoding (RMET) capacities in patients with acute, nonpsychotic MDD and in healthy, never-depressed, population controls. We assessed the severity of childhood adversities and then examined their impact on the RMET performance of depressed patients. To characterize the effect of childhood adversities further, we also evaluated the effect of physical, emotional, sexual abuse, and neglect on the response accuracy across the various RMET valences. We hypothesized that patients with depression will have poorer RMET accuracy than healthy controls, and that adverse childhood experiences will have a worsening effect on these deficits.

METHODS

Participants

Patients with MDD (N = 60) were recruited from the affective disorder unit of the Department of Psychiatry and Psychotherapy, University of Pécs. All patients fulfilled the DSM-5 diagnostic criteria of MDD (30). Inclusion criteria of the MDD group included: (1) age 18–55 years; (2) a diagnosis of MDD in a current major depressive episode (≥8 points on the Hamilton Depression Rating Scale (HAM-D), 21-item version (31). Because the clinical sample was recruited from the acute setting, the relatively long screening and testing procedure did not make it possible to investigate severely depressed participants. Exclusion criteria of the patient group were: current substance abuse or dependence (if the patient met diagnostic criteria he or she had to be abstinent for at least 2 years); current and lifetime psychotic symptoms, bipolar disorder, organic psychiatric disorders. Patients with any history of severe internal medical or neurological disorders, in addition, those with a history of head injury and with severe hearing or visual impairment; and an Intelligence Quotient (IQ) < 85 were also excluded. Although MDD is a common comorbid disorder in borderline personality disorder, considering the controversial data on RMET performances in patients with borderline personality disorder (meta-analyzed by 14, 32), MDD patients with comorbid borderline personality disorder were also excluded from this study.

The mean age of disease onset was 24.81 (±8.83) years. The mean duration of illness was 9.13 (±7.53) years with a range of 0.4–26 years. Fifty-seven (95%) patients with MDD received antidepressant medication (SSRI: 29; SNRI: 5; NaSSA: 12; bupropion: 2, agomelatine: 5, trazodone: 2; in combination with mood stabilizer: 3; in combination with low-dose atypical antipsychotic: 3).

The healthy control group (HC, N = 32) was matched in age, sex, and level of education. HCs were screened by a qualified psychiatrist to ascertain the absence of lifetime or family history of mental disorders; in addition, SCL-90 (33) was applied to rule out relevant subthreshold psychiatric symptoms in the potentially healthy individuals. Exclusion criteria for controls included a history of substance abuse in the past 24 months, a history of neurological disorders, a history of head injury with loss of consciousness for more than 30 min, an IQ < 85, and any learning difficulties. None of the healthy individuals took psychotropic medication. Four participants of the HC reached scores in the childhood trauma questionnaire (CTQ) in the moderate range and consequently, they were excluded from the study.

The local Research Ethics Committee of the University of Pécs approved the study design and protocol (Ethical Approval Nr.: 2015/5626) and all participants provided written informed consent.

Clinical Assessments

1. Diagnostic assessment: the current major depressive episode was assessed by a trained psychiatrist using the Structured Clinical Interview for DSM-5 disorders (SCID-5-CV and SCID-5-PD; 34, 35). Patients with MDD had nonexcluded comorbidities as follows: anxiety disorders (panic N = 7; social phobia N = 5; GAD N = 3; specific phobias N = 2), PTSD (N = 1), current OCD (N = 1), lifetime OCD (N = 2), lifetime alcohol use disorder (N = 3), lifetime sedatives, hypnotics, and anxiolytics use disorder (N = 4); cluster C personality disorders (dependent N = 4, avoidant N = 2).
2. Depression severity was evaluated using a multimethod approach. A trained clinician completed the 21-item Hamilton Rating Scale for Depression (HAM-D). The Beck Depression Inventory (BDI; 36) was applied as a self-assessment inventory.
3. Clinical data in patients with MDD (i.e., age at onset, length of illness, number of episodes, medications) were collected by clinical interviews, as well as by reviewing the affective disorder unit's charts and in- or outpatients' files.

Intelligence Quotient (IQ)

Full-scale IQ was measured using the Hungarian version of the Wechsler Adult Intelligence Scale (37, 38). Total IQ, performance intelligence quotient (PQ), and verbal intelligence quotient (VQ) values were calculated.

Assessment of Childhood Maltreatment

1. A senior psychiatrist (MS) conducted a structured interview with a focus on childhood adversities with all participants. Participants' answers during the interview were recorded on a preprinted interview sheet.
2. Childhood maltreatment was surveyed with the 28-item retrospective self-report questionnaire: the CTQ-short form (39) that assesses the severity of five types of maltreatment before the age of 18 years: physical abuse (PA), emotional abuse (EA), physical neglect (PN), emotional neglect (EN), and sexual abuse (SA). Each subscale consists of five items, all of them are evaluated on 5-point Likert scales. In our department, the internal consistency was excellent for the subscales: EA = 0.93, EN = 0.94, SA = 0.97, PA = 0.93, and good for the subscale PN = 0.77. During the data analysis,

CTQ raw scores were recoded into a two-level, binary variable by cut-off values on each subscale. Cut-off values were defined on the basis of a large normative sample consisting of 330 participants (university students and community sample).

3. Traumatic childhood experiences were also quantified with the self-report form of the Early Trauma Inventory—Self-Report (ETI) questionnaire (40). This 27-item questionnaire has 11 items for general traumatic experience, 5 PA items, 5 EA items, and 6 SA items that may have occurred before the age of 18 years. Each item can be answered with "yes" (scored as 1) or with "no" (coded as 0). For general traumas, factor analysis found three factors from which scores of the "dysfunctional family events" subscale (including witnessing family violence, separation of the parents, alcoholic parents) were entered into the further analyses.
4. Finally, interview results were compared with scores on trauma scales by a psychologist blinded to the patient. Discrepancies were discussed with the participants. In the case of unresolvable discrepancies, the participant was excluded from further analyses (N = 3).

On the basis of the measures listed above, the following variables were derived for further analysis:

1. Examining the impact of the prevalence of any trauma: when CTQ scores in any trauma dimension were at least in the moderate range, then, exposure to high childhood adversities (ACE) was assumed. Thus, patients were assigned to the *high-ACE MDD* subgroup if they had at least one type of moderate to severe child abuse and patients who did not experience any moderate to severe child abuse formed the *low-ACE MDD* subgroup.
2. Examining the impact of the specific traumas: All specific trauma measures were derived as binary measures from the scores of specific subscales (EN, EA, PN, PA, SA) of CTQ-SF. It was one if the scores were at least in the moderate range. Hence, patients with MDD were divided into *high-* and *low-EN, EA, PN, PA, and SA subgroups*.
3. Examining the impact of cumulative trauma: The variable "*number of traumas*" was calculated as a sum of the binary measures of specific types of adversities in CTQ and the "dysfunctional family events" score of the ETI. (Dysfunctional family score was one if the participant answered all of the three items with "yes.")

Assessment of ToM

Mental state decoding capacities were assessed using the revised version of the RMET (6). In RMET, a series of black-and-white photos presenting only the eye region is shown, and participants are instructed to pick one from four words presented simultaneously with the eyes to describe best the emotional state of the person in the photo. RMET consists of 36 trials and has been proven to be a valid measure of social sensitivity or mindreading (= mental state decoding). It shows a good test–retest reliability with no ceiling effect. Response accuracy and response time (in milliseconds) were digitally recorded. Based on Harkness et al. (7), items of the RMET were classified according to the emotional valences as positive (e.g., "friendly," N = 8), negative (e.g., "despondent," N = 12), or neutral (e.g., "pensive," N = 16).

Data Collection Procedures

During the first session, participants underwent diagnostic assessment and demographic interviews, then traumatic childhood experiences were assessed. In the second session (within 2 days), participants completed the RMET.

Statistical Analysis

First, initial exploratory *t* tests, chi-square tests, as well as bivariate correlations were conducted to explore associations between demographic and clinical variables, as well as ToM performances and the severity of childhood trauma. Subsequently, one-way ANCOVAs were performed to test between-subject differences in RMET scores with age, sex, and years of education as covariates.

Then, a series of 3 (group) × 3 (valence) mixed-model ANCOVAs with age, sex, and years of education as covariates was performed. To examine the effect of specific childhood adversities on the RMET accuracy, MDD patients were grouped on the basis of the specific subscales of the CTQ. First, RMET data of HC, as well as low- and high-ACE MDD groups were compared with an ANCOVA with age, sex, and years of education as covariates. Then, RMET data of healthy subjects and those of MDD patients with high- and low-EN, EA, PN, PA, as well as SA were entered into 3 (group) × 3 (valence) mixed-model multivariate ANCOVAs with age, sex, and years of education as covariates. Because of the overlap between the prevalence of some types of traumas, further multivariate ANCOVAs with controlling for other traumas were also performed.

To test the dose-response relationship between the cumulative effect of childhood adversities and the RMET performance across the various valences within the MDD group, the variable "number of traumas" was calculated. RMET total accuracy, as well as accuracies in the various valences, were entered as outcome variables in hierarchical multiple regression models. Predictor variables were entered in three steps: first, the predictive effect of the demographic variables (age, sex, and years of education) was tested, then, the severity of depression (i.e., BDI score) was added to the model, last the "number of traumas" was entered. Multicollinearity was tested with variance inflating factor (VIF), effect sizes were measured with η^2. The level of significance was set at $p = 0.05$.

RESULTS

Description of the Sample

Demographic and clinical variables, as well as IQ scores, are shown in **Tables 1** and **2**. Initial exploratory analyses (chi-square and *t* tests, as well as bivariate correlations, a series of ANOVAs) were conducted to find covariates for multivariate models (see the **Supplementary Material**).

TABLE 1 | Demographic and IQ data of the sample: there were no significant between-group differences.

	HC N = 32	MDD N = 60	2-group comparison statistics	low-ACE MDD N = 30	high-ACE MDD N = 30	3-group comparison statistics
Age (SD)	32.97 (7.75)	32.91 (8.39)	$t_{(90)} = 0.3$	33.15 (7.56)	32.7 (9.07)	$F_{(2,89)} = 0.02$
Females N (%)	20 (66%)	42 (75%)	$\chi^2_{(1)} = 0.67$	19 (73%)	23(%)	$\chi^2_{(2)} = 0.76$
Years of education (SD)	13.85 (2.15)	13.48 (2.49)	$t_{(90)} = 0.62$	14 (2.24)	13.10 (2.71)	$F_{(2,89)} = 1.28$
Tertiary education N (%)	14 (46.7%)	24 (42.85%)	$\chi^2_{(1)} = 0.12$	13 (50%)	11 (36.7%)	$\chi^2_{(2)} = 1.12$
IQ	111.6 (6.2)	110.4 (4.96)	$t_{(90)} = 1.31$	110.9 (4.79)	109.9 (5.14)	$F_{(2,89)} = 1.45$
VQ	110.5 (8.32)	111.7 (3.82)	$t_{(90)} = 0.71$	112.7 (5.08)	110.8 (7.31)	$F_{(2,89)} = 2.68$
PQ	112.7 (8.52)	109.6 (6.58)	$t_{(90)} = 1.86$	109.8 (7.39)	(109.4 (5.91)	$F_{(2,89)} = 1.42$

HC, healthy control group; MDD, Major depressive disorder group; ACE, adverse childhood experiences; high-ACE MDD, a subgroup of MDD patients with at least one type of any abuse or neglect in the moderate or severe range; low-ACE MDD, a subgroup of MDD patients with no abuse or neglect in the moderate or severe range. VQ, verbal intelligence quotient, PQ, performance intelligent quotient.

TABLE 2 | Clinical variables of the sample.

	HC N = 32	MDD N = 60	2-group comparison statistics	low-ACE MDD N = 30	high-ACE MDD N = 30	3-group comparison statistics
HAM-D (SD)	2.39 (1.54)	16.63 (2.71)***	$t_{(90)} = 27.99$	15.88 (2.2)	17.27 (2.97)	K-W stat = 2.81
BDI (SD)	4.43 (2.75)	23.02 (4.46)***	$t_{(90)} = 20.8$	22.23 (3.26)	24.7 (5.7)+	$F_{(2,89)} = 219.7$*** *post hoc*: HC< lowACE MDD < highACE MDD
BAI (SD)	6.13 (5.87)	20.05 (8.27)***	$t_{(90)} = 8.47$	19 (8.75-24)[a]	23.5 (16.75-27.25)[a]++	K-W stat = 47.41*** *post hoc*: HC < low-ACE MDD < high-ACE MDD
Age at onset (SD)	–	25 (17-39)	–	27 (19.13-32.25)	19 (16-19.13)	$U = 322$
Length of illness (years)[a]	–	8 (5.25-12)	–	8.5 (6-12)	8.0 (4.5-10)	$U = 331$
Recurrent depression N (%)	–	33 (55%)	–	12 (46.15%)	23 (76.6%)	$\chi^2_{(2)} = 6.94$
Number of episodes	–	2 (2)[a]	–	1 (1-3)[a]	2 (1-5)[a]++	$U = 255$
AD medication Yes (%)	–	58 (96.7%)	–	28	29	$\chi^2_{(2)} = 0.318$

*[a] medians (interquartile intervals) are presented; ***p < 0.001 compared to the HC; + p < 0.05 compared to the low-ACE MDD group; ++ p < 0.01 compared to the low-ACE MDD group.*

HC, healthy control group; MDD, Major depressive disorder group; ACE, adverse childhood experiences; high-ACE MDD, MDD patients with at least one type of abuse or neglect in the moderate or severe range; low-ACE MDD, MDD patients with no abuse or neglect in the moderate or severe range. K-W stat, Kruskal-Wallis statistic; U, Mann-Whitney U.

Child Abuse and Trauma Questionnaire Scores

Twenty-five MDD patients reported multiple adversities (41.67%), seven patients reported 2 (11.67%), eight 3 (13.33%), seven 4 (11.67%), and three 5 (5%) at least a moderate degree of childhood adversity. **Table 3** gives an overview of the frequency of the various forms of childhood adversities. A substantial co-occurrence of the specific types of childhood adversities was detected. Emotional abuse was strongly correlated with physical traumas and emotional neglect. The most common form of early-life adversity was emotional neglect followed by emotional abuse (95% of the emotionally neglected patients were also emotionally abused). All sexually abused patients were emotionally neglected, and 91.7% of them were at least moderately emotionally abused.

TABLE 3 | RMET data: comparison of healthy controls with patients with MDD.

	HC N = 32	MDD N = 60	ANOVA	ANCOVA[a]
RMET total score	74.13 (8.51)	70.46 (9.8)	$F_{(1,91)} = 3.18$ *ns*	$F_{(1,88)} = 3.29$ *ns*
RMET neutral valence	77.73 (12.49)	72.92 (11.83)	$F_{(1,91)} = 3.33$ *ns*	$F_{(1,88)} = 3.03$ *ns*
RMET positive valence	81.25 (16.50)	78.54 (16.61)	$F_{(1,91)} = 0.56$ *ns*	$F_{(1,88)} = 1.00$ *ns*
RMET negative valence	64.84 (10.94)	61.81 (14.82)	$F_{(1,91)} = 1.04$ *ns*	$F_{(1,88)} = 0.92$ *ns*

[a]Analysis of covariance with age, sex, and years of education as covariates. ns, not significant. Data are presented as mean (standard error). All RMET data are expressed as percent of the accurate answers.

HC, healthy control group; MDD, Major Depressive Disorder group; RMET, Reading the Mind in the Eyes Test.

86% of the physically neglected MDD patients were emotionally neglected, and 73.3% of them emotionally abused. Physical abuse was relatively rare, but emotional neglect and emotional abuse nearly always accompanied it (in 91% and 83% of the cases, respectively). Spearman's correlation analyses revealed that RMET total scores significantly correlated with CTQ total scores (rho = –0.232), CTQ PA (rho = –0.220), EA (rho = –0.234) EN (rho = –0.214), as well as RMET negative scores were significantly correlated with CTQ PA scores (rho = –0.269).

Mental State Decoding Accuracy: RMET Performances

RMET Accuracy in HC Versus Overall MDD Group

There were only trend level differences between the HC and MDD groups in the overall RMET accuracy (RMET total) scores (p = 0.077, η^2 = 0.034) and in RMET scores in neutral valences (p = 0.071, η^2 = 0.036). This between-group difference did not substantially change when controlling for age, sex, and years of education in the ANCOVA with overall RMET scores (p = 0.073, η^2 = 0.036) and with RMET scores in neutral valences (p = 0.085; η^2 = 0.034). (**Supplementary Material** presents all statistics of between-group comparisons of the RMET data.)

The Effect of Childhood Adversities on ToM Performance in MDD

One-way ANCOVA was conducted to determine a statistically significant difference between HC, low-ACE, and high-ACE MDD groups on RMET total scores controlling for age, sex, and years of education. There was a significant effect of group on RMET performance [$F_{(2,86)} = 3.87, p = 0.025, \eta^2 = 0.083$]. Post hoc Bonferroni correction indicated that the RMET total score of the high-ACE MDD group was significantly lower than that of the HC (**Figure 1**).

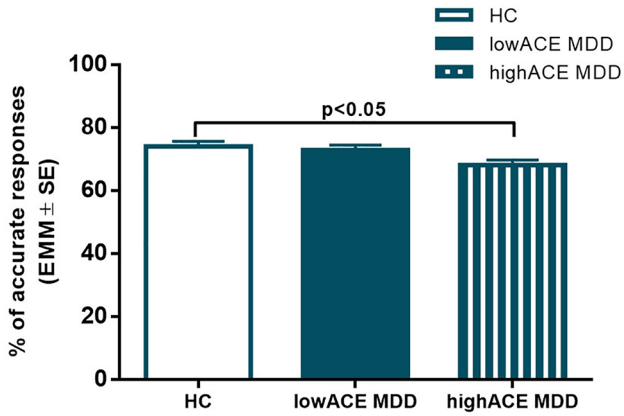

FIGURE 1 | Results of one-way ANCOVA with age, sex, and years of education as covariates. Estimated marginal means (EMM) are presented. Post hoc Bonferroni correction revealed that MDD patients with at least one type of moderate or severe childhood adversity performed significantly worse in RMET compared to healthy controls. ACE: Adverse Childhood Experience, HC: healthy control group, LowACE MDD: a subgroup of MDD patients without any moderate or severe childhood abuse or neglect, HighACE MDD: a subgroup of MDD patients with at least one type of moderate or severe childhood abuse or neglect.

The Effect of Specific Types of Adversities on RMET Valences

To test whether childhood adversities had any impact on the performance in RMET valences of depressed patients compared with nontraumatized HCs, 3 (group) × 3 (RMET valences: neutral, positive, negative) multivariate mixed-model ANCOVAs were run in each trauma dimension after controlling for age, sex, and years of education.

Emotional Maltreatment

Emotional Neglect (EN). The first model was a 3 (high-EN MDD, low-EN MDD, and HC groups) × 3 (RMET valences) mixed-model ANCOVA, with age, sex, and years of education as covariates. There was a significant main effect of group ($F_{(2,86)}$ = 4.32, p = 0.013, η^2 = 0.09), but the interaction of valence and group did not approach significance ($F_{(4,172)}$ = 1.03, p = 0.36, η^2 = 0.02). Pairwise comparisons showed a significant difference between HC and high-EN MDD (p = 0.047) (**Table 4**).

Emotional Abuse (EA). To explore the effect of emotional abuse, high-EA MDD, low-EA MDD, and HC groups were entered into the second 3 (group) × 3 (RMET valence) mixed-model ANCOVA with age, sex, and years of education as covariates. There was a significant main effect of group ($F_{(2,86)}$ = 5.24, p = 0.007, η^2 = 0.11), and a significant group × valence interaction ($F_{(4,172)}$ = 2.92, p = 0.023, η^2 = 0.06). Pairwise comparisons showed a significant difference between HC and high-EA MDD (p = 0.008), as well as between high-EA and low-EA MDD groups (p = 0.027) (**Table 4**).

Subsequently, one-way ANCOVAs were conducted by valence with age, sex, and years of education as covariates. In the negative valence, the main effect of group was significant ($F_{(2,86)}$ = 3.84, $p < 0.05$, η^2 = 0.082) (**Figure 2**), *post hoc* Tukey's multiple comparison test found that the high-EA MDD group significantly underperformed the HC in the RMET ($p < 0.05$). In addition, the low-EA MDD group had a significantly better response accuracy than the high-EA MDD group in the negative valence ($p < 0.05$). Similarly, in the positive valences, there was a significant main effect of group ($F_{(2,86)} = 5.19, \eta^2 = 0.108$), *post hoc* Tukey's revealed that both HC ($p < 0.05$) and low-EA MDD ($p < 0.01$) patients had greater response accuracy than the high-EA MDD group. However, there were no significant between-group differences in neutral valence (**Figures 3–5**).

Physical Maltreatment and Sexual Abuse

Physical Neglect (PN). Three (group) × 3 (RMET valence) mixed-model ANCOVA was performed with age, sex, and years of education as covariates. There was no significant group × valence interaction ($F_{(4,172)}$ = 2.01, p = 0.095, η^2 = 0.05), whereas a significant main effect of group ($F_{(2,86)}$ = 5.54, p = 0.005, η^2 = 0.11) could be detected. Pairwise comparisons showed that MDD patients with high-PN generally underperformed MDD patients with low-PN and HCs (p = 0.02 and p = 0.004, respectively) in RMET accuracy (**Table 4**).

TABLE 4 | The effect of specific childhood adversities on the response accuracy in RMET valences: results of 3(group) × 3(valence) mixed ANCOVAs.

	The main effect of group		Group x valence interaction	
	$F_{(df1, df2)}\eta^2$	η^2	$F_{(df1, df2)}\eta^2$	η^2
EN	**$F_{(2,86)}$ = 4.32***	**0.09**	$F_{(4, 172)}$ = 1.03	0.02
after controlling for:				
PA	**$F_{(2,85)}$ = 3.22***	**0.07**		
SA	**$F_{(2,85)}$ = 3.18 ***	**0.07**		
PN	$F_{(2,85)}$ = 0.74	0.02		
EA	$F_{(2,85)}$ = 1.01	0.02		
EA	**$F_{(2,86)}$ = 5.24****	**0.11**	$F_{(4, 172)}$ = 2,92*	0.06
after controlling for:				
PA	**$F_{(2,85)}$ = 4.17***	**0.09**	**$F_{(4,170)}$ = 4.79****	**0.1**
SA	**$F_{(2,85)}$ = 3.97***	**0.09**	**$F_{(4,170)}$ = 3.91***	**0.08**
PN	$F_{(2,85)}$ = 1.45	0.03	$F_{(4,170)}$ = 2.17	0.05
EN	$F_{(2,85)}$ = 1.18	0.04	$F_{(4,170)}$ = 2.35	0.05
PN	$F_{(2,86)}$ = 5.54**	0.11	$F_{(4, 172)}$ = 2.01	0.05
after controlling for:				
PA	**$F_{(2,85)}$ = 4.46***	**0.1**		
SA	**$F_{(2,85)}$ = 4.25***	**0.09**		
EA	$F_{(2,85)}$ = 2.61	0.06		
EN	$F_{(2,85)}$ = 2.41	0.054		
PA	$F_{(2,86)}$ = 2.09	0.05	$F_{(4, 172)}$ = 1.96	0.04
SA	$F_{(2,86)}$ = 2.79	0.06	$F_{(4,172)}$ = 0.49	0.001

*RMET accuracy data were subjected 3(group) × 3(valence) mixed model ANCOVAs with age, sex, and years of educations as covariates. In each analysis, the healthy control group was compared with two MDD groups. Patients with MDD were divided into 2 subgroups on the basis of their scores in CTQ subscales, e.g. MDD with high EA and low EA, etc. Then, other CTQ subscales were entered to control the effect of them. EA, EN, and PN substantially overlap, and mutually extinct the effect of each other on the RMET performance, EN, emotional neglect, EA, emotional abuse, PN, physical neglect, PA, physical abuse, SA, sexual abuse. *p<0.05; ** p<0.01. Effect sizes are presented as η^2. Statistically significant results are written is bold.*

Physical Abuse (PA). Three (group: HC, low-PA and high-PA) × 3 (RMET valence) mixed-model ANCOVA with age, sex, and years of education as covariates did not reveal any significant group × valence interaction ($F_{(4,172)}$ = 1.96, p = 0.103, η^2 = 0.04), or any main effect ($F_{(2,86)}$ = 2.09, p = 0.13, η^2 = 0.05).

Sexual Abuse (SA). Only 12 MDD patients reported sexual abuse during their childhood, and all of them were at least moderately emotionally neglected. 91.7% of them were emotionally abused, 50% of them experienced physical neglect, and 33% physical abuse. Three (group: HC, high-SA, and low-SA MDD) × 3 (RMET valence) mixed-model ANCOVA with age, sex, and years of education as covariates yielded only a trend level significance of the main effect of group ($F_{(2,86)}$ = 2.79, p = 0.067, η^2 = 0.06), and no group × valence interaction ($F_{(4,172)}$ = 0.49, p = 0.995, η^2 = 0.001).

Dose–Response Relationship Between the "Number of Traumas" and RMET Inaccuracies in the Entire MDD Group

To test the hypothesis that RMET inaccuracies are a function of the number of childhood adversities in the entire MDD group, three-stage hierarchical multiple regression analyses were conducted with RMET total scores, as well as with RMET scores in neutral, positive, and negative valences. Demographical variables (age, sex, and years of education) were entered at stage one. At stage two the severity of depression (measured with BDI) was entered, followed by "number of traumas" at stage 3.

Model 1

First, **RMET total scores** were entered as outcome variables. The best-fitting model for predicting RMET total scores were a linear combination of demographic variables (β_{age} = –0.08, t = –0.72, p = 0.477; β_{sex} = 0.35, t = 2.82, p = 0.007; β_{EDU} = 0.21, t = 1.722; p =

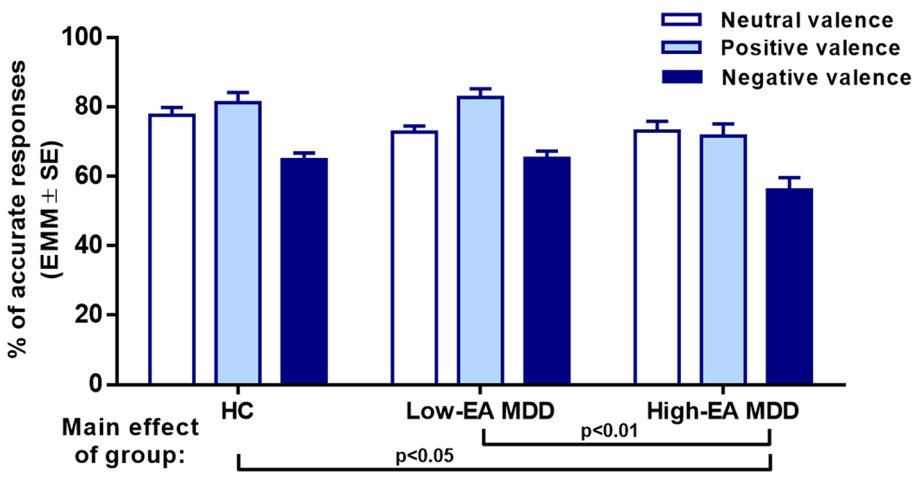

FIGURE 2 | Results of a 3 x 3 mixed ANCOVA with 'group' as an independent factor, and RMET valence as a within-subjects factor. Age, sex, and years of education were added as covariates. Estimated marginal means (EMM) are presented. There was a significant main effect of group and a significant interaction between group and valence. EA: Emotional Abuse, HC: healthy control group, Low-EA MDD: a subgroup of MDD patients without moderate or severe childhood emotional abuse, High-EA MDD: a subgroup of MDD patients with moderate or severe childhood emotional abuse.

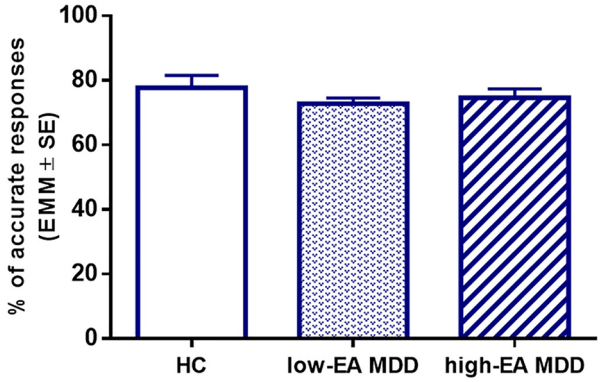

FIGURE 3 | Results of one-way ANCOVA with age, sex, and years of education as covariates. Estimated marginal means (EMM) are presented. There were no significant between-group differences. EA: emotional abuse, HC: healthy control group, Low-EA MDD: a subgroup of MDD patients without moderate or severe childhood emotional abuse, High-EA MDD: a subgroup of MDD patients with moderate or severe childhood emotional abuse.

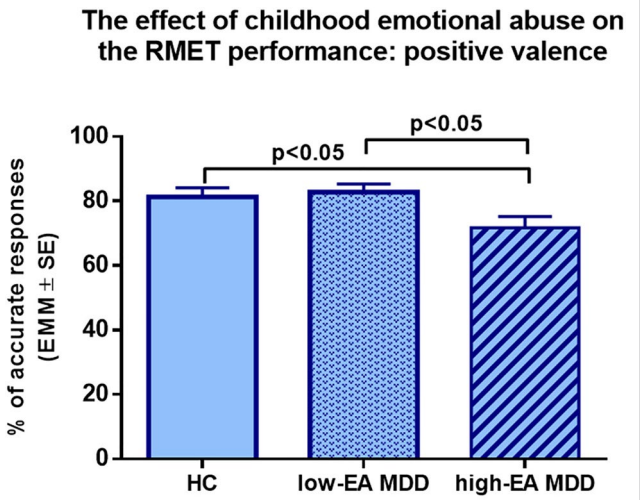

FIGURE 4 | Results of one-way ANCOVA with age, sex, and years of education as covariates. Estimated marginal means (EMM) are presented. MDD patients with moderate or severe childhood emotional abuse performed significantly worse in the positive valence compared to healthy controls and MDD patients without moderate or severe emotional abuse. EA: Emotional Abuse, HC: healthy control group, Low-EA MDD: a subgroup of MDD patients without moderate or severe childhood emotional abuse, High-EA MDD: a subgroup of MDD patients with moderate or severe childhood emotional abuse.

0.091), BDI score ($\beta_{BDI} = -0.14$; $t = -1.14$, $p = 0.258$), and number of traumas ($\beta_{NTRAUMAS} = -0.305$, $t = -2.52$, $p = 0.015$). Addition of the "number of traumas" did significantly improve prediction (R^2 change = 0.09, $F_{(1,54)} = 5.56$, $p < 0.022$) (**Table 5**).

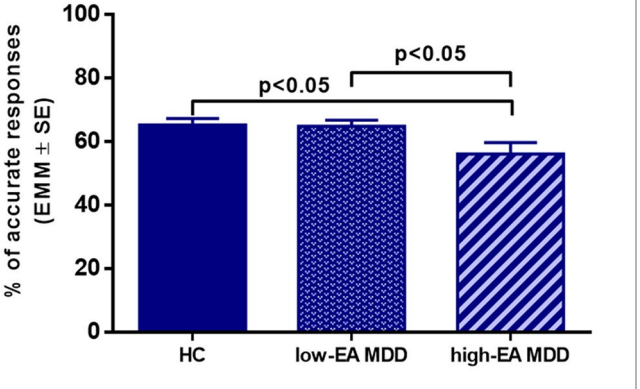

FIGURE 5 | Results of one-way ANCOVA with age, sex, and years of education as covariates. Estimated marginal means (EMM) are presented. MDD patients with moderate or severe childhood emotional abuse performed significantly worse in the negative valence compared to healthy controls and MDD patients without moderate or severe emotional abuse. EA: Emotional Abuse, HC: healthy control group, Low-EA MDD: a subgroup of MDD patients without moderate or severe childhood emotional abuse, High-EA MDD: a subgroup of MDD patients with moderate or severe childhood emotional abuse.

Model 2

Response accuracies in **RMET neutral valences** were entered as outcome variables. Predictive variables were the same as in Model 1. The best-fitting model was a linear combination of demographic variables ($\beta_{age} = -0.17$, $t = -1.4$, $p = 0.168$; $\beta_{sex} = 0.25$, $t = 2.02$, $p = 0.048$; $\beta_{EDU} = 0.36$, $t = 2.91$; $p = 0.005$). Adding BDI, as well as the number of traumas, did not significantly improve prediction (R^2 change = 0.05, $F_{(1,55)} = 3.64$, $p < 0.062$; R^2 change = 0.005, $F_{(1,54)} = 0.35$, $p < 0.556$, respectively) (**Table 6**).

Model 3

Next, response accuracies in **RMET positive valences** were entered in the hierarchical regression analysis as an outcome variable. Predictive variables were the same as in Models 1 and 2. Here, none of the predictors contributed significantly to the regression model (**Table 6**).

Model 4

Finally, response accuracies in the **RMET negative valences** were subjected to a hierarchical regression analysis as an outcome variable, whereas predictive variables were identical with those in previous models. Only stage 3, the addition of the number of traumas to the regression significantly improved prediction (R^2 change = 0.14, $F_{(1,54)} = 9.5$, $p < 0.01$), the "number of traumas" explained 38.7% of the variation in response accuracy in RMET negative valences, and it was the only significant predictor of the RMET performance in the negative valence (**Table 6**).

TABLE 5 | Summary of hierarchical regression analysis for variables predicting RMET total score.

Variable	*B*	SE	β	*t*	*R*	R^2	ΔR^2
Model 1: RMET total							
step1					0.37	0.14	0.14
age	-0.12	0.15	-0.1	-.8			
gender	6.23	2.83	0.28	2.2*			
years of education	1.13	0.5	0.29	2.29*			
step2					0.41	0.11	0.03
age	-0.12	0.15	-0.1	-.82			
gender	7.05	2.88	0.31	2.45*			
years of education	1.0	0.5	0.26	2.0			
BDI	-0.38	0.29	-0.17	-1.32			
step3					0.5	0.25	0.09
age	-0.10	0.14	-0.08	-0.72			
gender	7.74	2.78	0.35	2.82**			
years of education	0.83	0.49	0.21	1.72			
BDI	-0.31	0.28	-0.74	-1.14			
No. of traumas	-1.42	0.6	-0.31	-2.52*			

*N, 60, * p<.05; ** p<.01;*

BDI, Beck Depression Inventory score; RMET, Reading the Mind in the Eyes Test, total, total scores.

DISCUSSION

Main Findings

There was no difference in the mindreading abilities of the healthy controls and MDD patients. However, when we divided the MDD group into two subgroups, one with high and another with low levels of childhood adversities then, a significant difference emerged between the controls and the highly maltreated MDD subgroup in RMET performance. The main finding of our study is that MDD patients with at least moderate childhood neglect or emotional abuse are impaired in the RMET. Furthermore, MDD patients with childhood emotional abuse were significantly less accurate compared to healthy controls and MDD patients without emotional abuse in both the positive and negative valences of RMET. Finally, we found a dose-response relationship between the "number of traumas" and the RMET total scores and the scores of the RMET's negative valence.

A part of our results is in accordance with the findings of Rnic and co-workers (29), who also investigated the effect of childhood adversities on RMET in MDD. Similar to our present data, Rnic and co-workers found that the history of childhood emotional abuse in patients with MDD significantly worsened the RMET response accuracy. However, our study design differed from that of Rnic and co-workers because we examined only nontraumatized healthy participants. Perhaps due to our rigorous clinical assessment methods, we could identify only four HC individuals who were at least moderately traumatized and had no clinical or subclinical symptoms.

In contrast to the findings of Rnic and co-workers (29), we report here that both emotional and physical neglect had a negative impact on the patients' RMET performance. In our study, childhood adversities were assessed with CTQ which measures emotional neglect and physical neglect separately. The internal consistency of the physical neglect subscale was the lowest among the subscales, but it was still good (Cronbach's alpha = 0.77). In general, the exploration and assessment of early-life neglect are relatively difficult, as any kind of neglect has a hidden character (41). On the other hand, emotional neglect strongly intercorrelated with emotional abuse in our sample. As they almost entirely overlapped, it was not possible to investigate their effects separately. **Table 4** shows that the significant effects of both emotional and physical neglect disappeared after controlling for emotional abuse. Hence, we should carefully interpret and generalize our results with the effect of emotional abuse and neglect. Nonetheless, we can conclude that patients who were exposed to both emotional abuse and neglect performed significantly worse in the decoding of subtle emotional cues. To resolve controversies, we applied another approach and included the number of co-occurring types of early-life adversities (= "number of traumas") for each patient in the MDD group. We found a dose-response relationship between the "number of traumas" and RMET scores: multiple adversities during childhood had a negative effect on RMET total performance and on the performance in the negative valence.

The Impact of Depression Severity on the RMET Accuracy

Lee and co-workers (8) reported that severely depressed MDD patients' mental state decoding abilities were significantly worse than those of a healthy community sample, while patients with a mild/moderate MDD did not differ from that. In another study, patients with severe, psychotic MDD significantly underperformed the less severely depressed, nonpsychotic MDD patients in ToM tasks (10). While an additional study investigating less severely depressed individuals found that patients in a major depressive episode performed more accurate than healthy controls in the negative emotional valence of RMET (12). Furthermore, increased sensitivity to social stimuli was observed in a population sample with dysphoria (measured with

TABLE 6 | Summary of hierarchical regression analyses for variables predicting RMET scores.

Variable	*B*	SE	β	*t*	*R*	R^2	ΔR^2
Model 2:							
RMET neutral							
step1					0.43	0.18	0.18
age	-0.24	0.17	-0.17	-1.4			
sex	6.73	3.33	0.25	2.02*			
years of education	1.69	0.58	0.36	2.91**			
step2					0.48	0.23	0.05
age	-0.25	0.17	-0.17	-1.44			
sex	8.11	3.33	0.3	2.43**			
years of education	1.47	0.58	0.31	2.54**			
BDI	-0.64	0.33	-0.24	-1.91			
step3					0.49	0.24	0.01
age	-0.24	0.17	-0.17	-1.4			
sex	8.24	3.38	0.31	2.47*			
years of education	1.43	0.6	0.3	2.41*			
BDI	-0.62	0.34	-0.23	-1.84			
No. of traumas	-0.27	0.73	-0.07	-0.59			
Model 3:							
RMET positive							
step1					0.18	0.03	0.03
age	0.22	0.27	-0.11	-0.82			
sex	5.24	5.09	0.14	1.03			
years of education	0.47	0.89	0.07	0.53			
step2					0.18	0.03	0.00
age	0.22	0.27	0.11	0.82			
sex	5.06	5.26	0.12	0.96			
years of education	0.50	0.91	0.08	0.55			
BDI	0.09	0.53	0.23	0.16			
step3					0.26	0.07	0.03
age	0.24	0.27	0.12	0.89			
sex	5.81	5.25	0.15	1.11			
years of education	0.32	0.92	0.05	0.35			
BDI	0.16	0.53	0.04	0.3			
No. of traumas	-1.53	1.13	-0.2	-1.35			
Model 4:							
RMET negative							
step1					0.23	0.05	0.05
age	-0.18	0.23	-0.1	-0.76			
sex	6.21	4.49	0.18	1.38			
years of education	0.82	0.78	0.14	1.06			
step2					0.25	0.06	0.01
age	-0.18	0.24	-0.1	-0.76			
sex	6.96	4.62	0.21	1.51			
years of education	0.71	0.8	0.12	0.88			
BDI	-0.35	0.46	-0.1	-0.75			
step3					0.45	0.2	0.14
age	-0.15	0.23	-0.08	-0.67			
sex	8.38	4.32	0.25	1.94			
years of education	0.36	0.75	0.06	0.48			
BDI	-0.21	0.43	-0.06	-0.49			
No. of traumas	-2.88	0.93	-0.39	-3.08**			

*N, 60, *p<.05; **p<.01; BDI, Beck Depression Inventory score; RMET, Reading the Mind in the Eyes Test, neutral, scores in the neutral valence; positive, scores in the positive valence; negative, scores in the negative valence.*

BDI): a college sample with dysphoria had superior accuracy in decoding mental states compared to a nondysphoric group (7). Greater accuracy in RMET performance was reported in depressed and nondepressed women with a maternal history of depression (1), and in patients with past MDD (11) indicating that both an increased vulnerability for depression and a previous depressive episode can significantly enhance the capacity to accurately identify subtle emotional cues.

Our findings are in harmony with the results reviewed above. We examined a relatively homogeneous group of MDD patients with mild or moderate depression, and we rigorously controlled the HC group for subclinical symptoms. When we compared the

entire MDD group with HC, we found no significant between-group differences in RMET performances. However, significant between-group differences appeared when we analyzed the MDD subgroup with a high level of early adversities (= high-ACE MDD group) separately. Moreover, BDI scores were entered into our final hierarchical regression model. The "number of traumas" significantly predicted the inaccuracies in RMET total scores and in the negative valence even after controlling for severity of current depression. Hence, childhood adversities were much stronger predictors of RMET inaccuracies than the severity of depression in our mildly/moderately depressed MDD group.

The Effect of Early-Life Adversities: Possible Underlying and Mediating Factors

Exposure to childhood adversities (particularly to emotional abuse) is known to result in increased reactivity of the amygdala which makes the affected individuals more vulnerable to negative psychosocial experiences during adulthood (42). In addition, early-life stress results in dysregulation of the hypothalamus-pituitary gland-adrenal axis, which makes the individual more susceptible to stressful life situations (43, 44). Thus, we can assume, that negative social cues evoke increased stress, particularly in those MDD patients who were exposed to emotional abuse (e.g. anger, hostility) as a child, which might worsen their mental state decoding abilities particularly in the emotional valences. These assumptions are in line with the results of Hentze and co-workers (45) who investigated chronically depressed patients' regional brain activations during an affective ToM task and found significantly increased amygdala activation in patients with childhood maltreatment. Altough no brain-imaging study has examined the brain activations during ToM tasks specifically in adult MDD patients with early adversities (46), we can speculate that MDD patients who were exposed to early life stress might react with a more pronounced amygdalar activation that might impair their RMET accuracy.

A recent study, measuring the peak amplitudes of N170 face-sensitive visual ERP component responses to emotional faces in asymptomatic adults with or without childhood trauma, suggested that exposure to childhood trauma was associated with a failure to differentiate between threat-related and nonthreat-related emotional stimuli (47). These findings indicate that early-life stress may be related to a generalized emotional hyper-responsibility to emotional cues, which in turn makes it difficult to recognize accurately the emotional content of them (47). Moreover, it was found that early-life parental maltreatment (particularly physical abuse but also neglect to a certain extent) negatively influenced RMET accuracy in a large, internet-based, adult community sample (17). Hence, we can assume, that the emotional hyper-reactivity and the difficulties of emotional regulation—due to the increased amygdala activity—make it more challenging for asymptomatic individuals with early adversities to mirror adequately the emotional contents and identify them. In a recent functional MRI study, a regional hyperactivation of the pars triangularis was detected in youth with sexual abuse and emotional maltreatment during the RMET task, which was interpreted as a compensation of the impaired mirroring capacities of the participants (48). In addition, the authors concluded that the combination of sexual abuse and emotional maltreatment can be particularly toxic. This corresponds with our results on the negative effect of the co-occurrence of various maltreatment types on RMET.

Clinical Relevance of the Findings

MDD patients with childhood adversities have an elevated risk of developing recurrent or chronic depressive episodes, and are more often therapy-resistant (e.g., meta-analyzed by 49–51). According to our findings, early-life emotional abuse, mostly if they co-occurred with physical and emotional neglect resulted in impaired mental state decoding capacities in MDD. In particular, the decoding of emotional cues (both negative and positive) was inaccurate.

Considering that early-maltreated MDD patients have a reduced ability to decode social cues, one can predict that they experience significantly higher levels of stress in social situations, especially when they need to assess emotional cues. This may worsen their social withdrawal, as well as increase their existing vulnerability to stressful life events. All these are in harmony with the common clinical and epidemiological experience that early-life adversities worsen the outcome and the course trajectory of MDD. In sum, when planning their therapy, clinicians need to consider the effect of childhood adversities, they should count on therapy resistance or chronicity, and should strive to remediate these patients' ToM and mentalizing abilities.

Limitations of the Study

The major limitation of our study was that no never-depressed individuals with at least a moderate degree of abuse or neglect were entered in the analysis. On the basis of the literature, we aimed to control the effect of subclinical depressive symptoms, therefore, inclusion criteria were relatively strict. The very few HC subjects with relevant childhood adversities (N = 4) were excluded from the analysis because their number was too low to analyze them as a separate group. Further analyses are necessary to test the effect of adverse childhood experiences on the mental state decoding abilities in healthy individuals, to disentangle the effect of MDD and that of childhood adversities. Hence, we should very carefully interpret our findings on the role of childhood adversities.

Furthermore, childhood emotional abuse, emotional neglect, and physical neglect were associated with inaccurate RMET performance in MDD patients, whereas no effects were observed for physical and sexual abuse. Nevertheless, the prevalence of physical and sexual abuse was relatively low in our MDD sample. As a consequence of it, the differential effects of specific types of early life adversities might also be a consequence of differential statistical power. In addition, childhood adversities were measured retrospectively, during an acute depressive episode. Therefore, recall biases induced by the current depression could not be fully ruled out (52).

Another limitation is that we did not include the Beck Anxiety Inventory (BAI) scores in our multiple regression models. The

severity of anxiety and depression strongly overlapped in our MDD sample (Spearman's rho = 0.613, $p < 0.001$), so we interpreted it as a symptom of the current depression. Nevertheless, our primary correlation analyses revealed a strong correlation between BAI scores and the severity of childhood adversities (rho = 0.341, $p < 0.008$). Anxious depression is a common subtype of MDD among patients with childhood adversity. It has been recently reported that anxious depression in patients with early-life adversities sensitizes the glucocorticoid receptors (53).

The effects of neurocognitive functions and verbal abilities were not extensively examined in our study. (We assessed IQ, VQ, and PQ). Nevertheless, we found that the total RMET response accuracy and that in the neutral valence correlated with the years of education (Pearson's r = 0.323, $p < 0.001$; and r = 0.411, $p < 0.001$, respectively). There was a weaker, but still significant correlation (Pearson's r = 0.262, $p < 0.05$; r = 0.305, $p < 0.01$, respectively) between IQ and RMET performance as well. Although Baron-Cohen and co-workers (6) reported that the intelligence did not contribute to the processes involved in RMET, a recent meta-analysis involving 77 studies found a small positive correlation (r = 0.24) between IQ and RMET performance without any significant difference between the effects of verbal and performative intelligence (54). In addition, several studies found a correlation between executive functions and ToM capacities in MDD (meta-analyzed by 4). In the future, more extensive assessment of neurocognitive functions, verbal fluency, and vocabulary are necessary to evaluate their exact impact on RMET accuracy, especially on that in the neutral valence, as well as the way in which childhood adversities can influence it.

Finally, we did not control the effect of antidepressant medication. The majority of our clinical MDD sample (95%) was medicated and took antidepressants from various classes. Therefore it was not possible to form homogeneous medication groups for further analysis.

CONCLUSIONS

In sum, our present findings document that childhood adversities impair the mental state decoding abilities of MDD patients during the acute episode. Multiple adversities in general, but particularly the co-occurrence of emotional abuse and neglect, were found to have a more disadvantageous effect with a dose-response character on mental state decoding capacities in MDD. Further (preferably follow-up) research is needed to clarify the exact underlying mechanisms and therapeutic interventions beneficial for this subgroup of MDD patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The local Research Ethics Committee of the University of Pécs approved the study design and protocol (Ethical Approval Nr.: 2015/5626) and all participants provided written informed consent

AUTHOR CONTRIBUTIONS

MS, BC, and NN conceived and designed the study. NN, EC, EL, and MG collected the data. Data analysis was performed by MS with special assistance from NN, EC, MG, and EL. MS wrote the manuscript. TT provided supervision and had helpful comments on the interpretation of the data. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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.2019.00867/full#supplementary-material

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Cognitive Behavioral Therapy for Antenatal Depression in a Pilot Randomized Controlled Trial and Effects on Neurobiological, Behavioral and Cognitive Outcomes in Offspring 3–7 Years Postpartum: A Perspective Article on Study Findings, Limitations and Future Aims

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Purpose of Article: In a previous pilot randomized controlled trial including 54 pregnant women with depression, maternal mood improved after Cognitive Behavioural Therapy (CBT) compared to treatment as usual (TAU), showing medium to large effect sizes. The effect persisted up to 9 months postpartum, with infant outcomes also showing medium to large effects favoring CBT in various child domains. This perspective article summarizes the results of a follow-up that was performed approximately 5 years later in the same cohort, assessing the effects of antenatal Cognitive Behavioural Therapy for depression and anxiety on child buccal cell DNA-methylation, brain morphology, behavior and cognition.

Findings: Children from the CBT group had overall lower DNA-methylation compared to children from the TAU group. Mean DNA-methylation of all *NR3C1* promoter-associated probes did not differ significantly between the CBT and TAU groups. Children from the CBT group had a thicker right lateral occipital cortex and lingual gyrus. In the CBT group, Voxel-Based-Morphometry analysis identified one cluster showing increased gray matter concentration in the right medial temporal lobe, and fixel-based analysis revealed reduced

fiber-bundle-cross-section in the Fornix, the Optical Tract, and the Stria Terminalis. No differences were observed in full-scale IQ or Total Problems Score. When the total of hypotheses tests in this study was considered, differences in DNA-methylation and brain measurements were no longer significant.

Summary: Our explorative findings suggest that antenatal depression treatment decreases overall child DNA-methylation, increases cortical thickness, and decreases white matter fiber-bundle cross-section in regions involved in cognitive function and the stress response. Nevertheless, larger studies are warranted to confirm our preliminary conclusion that CBT in pregnancy alters neurobiological outcomes in children. Clinical relevance remains unclear as we found no effects of antenatal CBT on child behavior or cognition (yet).

Keywords: depression, anxiety, pregnancy, Cognitive Behavioural Therapy, neurodevelopment, programming, offspring

INTRODUCTION

Antenatal depression occurs in approximately one out of 10 pregnant women (1–3). Pregnant women with depression are less inclined to take good care of themselves, which is reflected by poorer eating habits, more substance abuse and avoidance of prenatal care (4). Also, antenatal depression is a predictor of postnatal depression, which on itself is a detrimental condition that is accompanied by many health risks for both mother and child (5). Moreover, not only the woman herself is affected by the disease, her unborn child may be also. Studies have shown that children who were born to mothers affected by depression during pregnancy were more often born prematurely and had lower birth weights compared to children born to mothers without depression in pregnancy (6, 7). Antenatal depression has also been linked to poorer neonatal neurodevelopmental outcomes such as state-regulation, habituation, regulatory behaviors and sleep problems (8–10), a more difficult temperament in childhood (11), and poorer cognition both in the neonatal period (12) and in childhood (13). The evidence for an association between antenatal depression and offspring behavioral and emotional problems differs among studies, and also seems dependent of the age when the child assessment took place. Whereas one study showed that antenatal depression was associated with both higher internalizing behavior and more behavioral problems at the age of 3 (14), others did not report such an association at the same age (15, 16). However, at the age of 10, children who had been exposed to maternal depression *in utero*, had higher scores on total emotional and behavioral problems (17), and in cohorts of children aged 16, elevated externalizing behavior (18, 19), and conduct problems (20) were observed in those who were born to mothers with antenatal depression. Also, in (pre)adolescence, a higher likelihood for depression and anxiety symptomatology was seen in offspring from mothers who were depressed when they were pregnant (21–23). In some studies, the described associations between antenatal depression and offspring neurodevelopment are attenuated after postnatal maternal depressive symptoms are taken into account (13, 15), whereas other studies that took into account many important confounding do point to an independent contribution of the antenatal period to offspring neurodevelopmental disorders and psychopathology (24, 25). During the past decade, the number of studies that carefully address potential confounding factors has increased [reviewed by Van den Bergh et al. (26)], with some studies also including paternal symptoms of depression, to address the issue of genetic inheritance of vulnerability to psychopathology (27). Another study that also nicely demonstrated that effects of antenatal depression on offspring development are (at least partly) independent of genetic traits, is a study in which women became pregnant by *in vitro* fertilization (IVF), either with their own or through a donor oocyte, after which associations between prenatal depressive symptoms and behavioral problems in the children were analyzed. They showed that, although associations with ADHD were observed only in genetically related children, depression symptoms were related to conduct disorder in both genetically related and unrelated children (28).

Several potential biological mechanisms have been suggested to link maternal depression exposure in *utero* to an altered offspring neurodevelopment. A frequently postulated explanation is that as a result of the depressive state in a pregnant woman, cortisol levels increase through chronic activation of the hypothalamic–pituitary–adrenal (HPA) axis, which then crosses the placental barrier and negatively affects fetal brain development, which has clearly been shown in animal studies (29, 30). However, evidence on associations between depression during pregnancy and cortisol values in humans have been inconsistent, suggesting the involvement of more complex or additional underlying mechanisms that are simultaneously activated in the mother, as a result of the depression (31). Physiological systems that may be involved in linking maternal depression to fetal neurodevelopment besides the neuroendocrine system (HPA-axis), are the autonomic nervous system (32), the cardiovascular system (33, 34) and the immune system (35–37). As a consequence of the alterations in maternal biochemical processes described above, fetal (neuro) development may be altered. On a cellular level, epigenetic alterations of fetal DNA may occur, potentially leading to

altered gene expression and protein synthesis and eventually, differences in phenotype. Studies in humans have shown that antenatal depression is associated with differential methylation of offspring genes involved in neurodevelopment or psychopathology, with the glucocorticoid receptor gene (*NR3C1)* showing the most consistent result of increased methylation after prenatal maternal depression exposure (38–41). The glucocorticoid receptor (GR) plays a pivotal role in the negative feedback loop of the HPA-axis, eventually dampening the stress response and cortisol release. Hence, HPA-axis sensitivity and stress responsivity may be permanently altered by prenatal depression through increased fetal *NR3C1* methylation and lower GR density, which eventually may lead to a heightened susceptibility for psychopathology such as depression and anxiety. Brain regions that are known to be involved in psychopathology and neurodevelopment include the amygdala, hippocampus, and cortical structures located primarily in the (pre)frontal and medial lobe. Morphological characteristics of the brain in children visualized with MRI techniques have been shown to be associated with prenatal exposure to depression. For example, thinner frontal and medial cortices and larger amygdala's have been observed in children from mothers who had higher symptoms of depression in pregnancy compared to children from non-depressed mothers (42, 43). Besides the volume and shape of the brain, the way the brain is 'wired' may also be affected by i*n utero* exposure to maternal depression, through altered development, migration or maturation of neuronal axons resulting in adverse connectivity patterns or functionality between brain regions. Studies have observed greater functional connectivity of mainly the amygdala with surrounding brain regions in children who had been exposed to increased maternal depressive symptomatology before birth (44, 45). Also, a higher mean white matter diffusivity, roughly reflecting a decrease in white matter integrity, of the uncinated fasciculus and the cingulum bundle has been described in children after prenatal exposure to maternal depression, although the latter was attenuated by paternal prenatal depressive symptoms (46).

The evidence from longitudinal studies on child neurobiological outcomes after prenatal exposure to maternal depression suggests a direct independent contribution of the antenatal period to child neurodevelopment. However, confounding due to genetic influences or other inherent maternal traits that are associated with both depression and child neurodevelopment, are challenging, if not impossible, to consider. The only way to reliably establish causality, is by performing randomized controlled trials (RCT) (47, 48). Nevertheless, experimental study designs in humans are difficult, as depression is a non-random event and cannot be 'forced' upon someone. However, it is possible to assess whether a reduction in depression symptoms in pregnancy, as a result of a randomly allocated treatment in a group of depressed pregnant women, leads to changes in the assumed neurobiological systems in the desired directions, as well as to improvements in neurodevelopmental outcomes in children, thereby providing more conclusive evidence on the effects of (untreated) depression on child neurodevelopment.

To address this pivotal gap in research, the *Beating the Blues before Birth* cohort study was developed at the Parent Infant Research Institute in Melbourne, Australia. A pregnancy-adjusted Cognitive Behavioral Therapy (CBT) for depression and anxiety during pregnancy was developed and assessed in a feasibility study and pilot randomized controlled trial (49). The program consisted of seven individual sessions of CBT and one session in which the partner was included. Women aged 18 years or older, less than 30 weeks pregnant, and with a depressive disorder were included. Recruitment of women occurred *via* screening programs at the Northern Hospital and Mercy hospital for women, as well as *via* various health services and professionals, and the private sector, to which the program was advertised widely. If a woman was suspected for a clinical depression and met inclusion criteria, the Edinburgh Postnatal Depression Scale [EPDS; (50)] was conducted, and if the woman scored 13 points or higher on the scale, she was asked for her consent to participate in the study. Women who consented were referred to a psychologist to participate in a Structured Clinical Interview (51), according to DSM-IV criteria to yield a diagnosis of minor or major depression, or adjustment disorder with mixed depression and anxiety (52). Concurrent major psychiatric disorders, comorbid axis I disorders or medical conditions that were likely to interfere with study participation, risk requiring crisis management in case of very severe symptoms and suicidal ideation, participation in other psychological programs and significant difficulty with English were the exclusion criteria. Symptom severity of depression and anxiety was measured by use of the Beck Depression (BDI-II) and Anxiety (BAI) Inventories, both 21-item clinical instruments with well-established psychometric properties (53, 54). The distributions of the different types of depression were: major depression 72%, minor depression 9%, adjustment disorder 19%. Women were randomized to receive CBT or Treatment as Usual (TAU). TAU meant that women were either referred to their GP for further evaluation and management or that they would be case managed by their midwife, as would usually have happened in routine practice. The BDI-II and BAI were assessed before randomization, 9 weeks after randomization and again at 9 months postpartum. Following the program, substantial improvements in depression and anxiety symptoms in favor of the CBT group were observed. Depression symptoms (BDI-II) dropped, on average, from the 'severe' range (BDI-II = 30.07) to the 'minimal' range (BDI-II = 12.81) in the CBT group, whereas the drop in the TAU group was more modest ('*Cohens' d* = 0.53, 95% CI = 0.26 to 0.79). On average, anxiety symptoms decreased from the 'moderate' (BAI = 22.37) to the 'mild' range (BAI = 10.40) in the CBT group, but remained relatively elevated in the TAU group (d = 0.67, 95% CI = 0.33 to 1.01) (49). At 9 months, treatment effects had maintained. Moreover, infant assessment at 9 months postpartum showed favorable outcomes on the 'problem solving' (d = 0.72, 95% CI = 0.36 to 1.08) and 'communication' (d = 0.53, 95% CI = 0.27 to 0.80) domain in the Ages and Stages Questionnaires [ASQ; (55)], as well as large effects favoring the CBT group on the subscales 'recovery from distress' (d = 1.08, 95% CI = 0.54 to 1.62), 'high intensity pleasure' (d = 0.83, 95% CI = 0.42 to 1.24) and 'negative affectivity' (d = 0.84, 95% CI = 0.42 to 1.26) on the Revised version of the Infant

Behaviour Questionnaire [IBQ-R;(56)] (49). At a 2-year follow-up, significant treatment effects were found on the Parenting Stress Index, with lower scores on the 'total score' (d = 1.44, 95% CI = 0.41 to 2.46), 'child domain' (d = 0.96, 95% CI = 0.16 to 1.75) and 'adaptability' (d = 0.88, 95% CI = 0.10 to 1.64) subscales, indicating a lower number of child characteristics that may contribute to overall stress in parents in the CBT group. No effects of treatment on motor or cognitive development and behavioral problems were found at the age of 2 (57).

STUDY DESCRIPTION

Current Perspective Article

Approximately 5 years postpartum a follow-up study to the original *Beating the Blues before Birth* trial was performed. In this study, we evaluated neurobiological alterations including offspring DNA methylation, morphological and microstructural brain properties, as well as behavioral and cognitive performance in 5-year old offspring of mothers participating in the *original t*rial. Findings on behavioral, cognitive, neurobiological and epigenetic outcomes have all previously been reported on in separate publications. In the present paper, we aim to (1) provide an overview of the most important outcomes of the follow-up study, (2) place these findings on different outcomes in a broader perspective, (3) reflect on strengths and limitations, and (4) provide recommendations for future studies on this clinically relevant topic of treating antenatal depression and studying outcomes in the offspring.

Follow-Up At Five Years of Age

The childhood follow-up study was initiated in 2016, aiming to study the effect of maternal antenatal CBT for depression on offspring neurobiological, behavioral and cognitive outcomes. All women who had participated in the original RCT with known contact details were re-contacted and invited to participate in a follow-up study of their children approximately 5 years postpartum. Baseline characteristics of mothers at enrollment and birth outcomes were retrieved from the original study files. A questionnaire on current sociodemographic characteristics (including maternal age, marital status, family income and highest completed education of the mother, child age, gender, gestational age and weight at birth) was completed by mothers. Mothers also completed the BDI-II and BAI for current symptoms of depression and anxiety. Children donated buccal swabs for DNA extraction and DNA methylation profiling. Differential DNA methylation was calculated (1) on a genome-wide level, (2) on 16 a *priori* selected genes that had been studied in association with prenatal exposure to maternal stress, and (3) on the promoter-associated probes on *NR3C1*. We derived measures of child brain gray matter density, volume and cortical thickness and white matter tract fiber densities and cross-sections from anatomical and diffusion weighted Magnetic Resonance Image (MRI)-scans. Mothers additionally completed the Child Behaviour Checklist [CBCL; (58)], and were invited to visit the clinic for a cognitive assessment with their child performed by a trained researcher by use of the Wechsler Preschool and Primary Scale of Intelligence, third edition [WPPSI-III; (59)].

Statistical Analyses

DNA methylation data was analyzed in statistical software package 'R'. Anatomical weighted brain MRI scans were analyzed using statistical software packages Freesurfer and SPSS IBM version 24 to analyze subcortical volumes and cortex thicknesses, and Statistical Parametric Mapping (SPM) to analyze Voxel Based Morphometry. Connectivity-Based Fixel Enhancement was used to analyze diffusion-weighted images to estimate fiber bundle orientation, fiber bundle cross-section and fiber bundle density. Behavioral and cognitive data was analyzed using SPSS IBM version 24. We compared all outcomes between children from the CBT and the TAU group for effect sizes and confidence intervals. The results are presented as explorative and hypothesis-generating in view of the low numbers and thereby low statistical power.

RESULTS

Participants

Of the originally 54 participating women, 45 were located and invited for participation. 24 families re-consented to the 5-year follow-up, 12 from the CBT and 12 from the TAU group. Baseline data was retrieved from all 24 mother–child pairs. Missing data included post-treatment BDI-II and BAI scores of two women from the TAU group. All 24 women completed a questionnaire on current sociodemographic information and completed the CBCL. Completed CBCL questionnaires were sent to us by mail by five women who were unable to visit the clinic for an additional cognitive assessment with their child. A total of 19 mothers and their children visited the clinic, of which all children participated in a cognitive assessment (WPPSI-III) (59). Twenty three mother–child pairs participated in the study of DNA methylation profiles, including all 19 who visited the clinic. An additional four mothers consented to send a buccal swab from their child using a pre-paid envelop sent to their homes. MR brain imaging was attempted in all 19 children who visited the clinic. The MRI data of three subjects had to be excluded from the analysis, because of significant motion artefacts, resulting in a total of 16 mother–child pairs included in the MRI study. **Table 1** shows the baseline characteristics of all women participating in the original trial, and those participating in respectively the DNA methylation study, the MRI study, the behavioral questionnaire and the cognitive assessment. We did not perform statistical tests to assess baseline characteristic differences according to the CONSORT statement (60). Women who participated in any of the follow-up's were on average older, more often highly educated, and had somewhat lower depression and anxiety symptoms scores, compared to the total sample from the original trial.

DNA Methylation

Children from the CBT group had overall lower DNA methylation compared to children from the TAU group

TABLE 1 | Baseline characteristics of all participants in a trial evaluating an antenatal cognitive depression treatment on offspring neurobiological, behavioral and cognitive outcomes.

Baseline demographic	All Participants		Epigenetics 5 y		MRI 5 y		Behavior 5 y		Cognition 5 y	
	CBT (n = 28)	TAU (n = 26)	CBT (n = 12)	TAU (n = 11)	CBT (n = 8)	TAU (n = 8)	CBT (n = 12)	TAU (n = 12)	CBT (n = 11)	TAU (n = 8)
T1 level depression (BDI-II), mean (SD)	30.8 (9.5)	30.5 (8.9)	29.6 (9.5)	29.5 (10.4)	29.8 (11.3)	26.4 (10.1)	29.6 (9.5)	29.2 (10.0)	29.4 (10.0)	27.6 (11.6)
T1 level anxiety (BAI), mean (SD)	22.8 (10.0)	21.2 (10.2)	19.2 (9.0)	19.3 (7.1)	18.8 (9.7)	17.5 (6.4)	18.2 (9.0)	19.7 (6.9)	20.0 (9.0)	18.8 (7.4)
T2 level depression (BDI-II), mean (SD)	13.0 (9.8)	17.4 (9.8)	13.0 (10.0)	17.6 (9.0)	12.0 (10.7)	18.3 (6.2)	13.0 (10.0)	18.1 (8.5)	11.9 (9.8)	21.5 (5.9)
T2 level anxiety (BAI), mean (SD)	10.6 (7.6)	16.7 (11.8)	11.6 (9.9)	15.3 (7.1)	11.6 (10.9)	15.5 (8.0)	11.6 (9.9)	16.2 (7.2)	11.6 (10.4)	17.5 (6.6)
T3 level depression (BDI-II), mean (SD)	–	–	16.1 (13.3)	14.9 (11.2)	15.0 (15.8)	15.6 (10.3)	16.1 (13.3)	14.6 (10.7)	14.7 (13.6)	14.0 (10.5)
T3 level anxiety (BAI), mean (SD)	–	–	11.3 (8.9)	10.9 (10.2)	11.1 (9.5)	11.9 (11.3)	11.3 (8.9)	10.3 (9.9)	10.2 (7.5)	11.3 (10.6)
T1 Maternal age in years, mean (SD)	32.9 (5.9)	31.0 (5.8)	33.7 (5.7)	33.6 (5.2)	33.3 (6.4)	34.3 (5.6)	33.7 (5.7)	33.3 (5.1)	33.7 (5.7)	35.8 (3.3)
T1 Gestational age in weeks, mean (SD)	19.9 (7.7)	21.0 (6.0)	18.3 (7.2)	19.0 (5.5)	16.6 (7.2)	20.0 (5.1)	18.3 (7.2)	19.5 (5.5)	18.7 (7.3)	18.9 (5.3)
T1 Antidepressant use (%)	7.1	22.7	0	11.1	0	12.5	8.3	8.3	9.1	0
T1 Marital status (%)										
–Married	57.7	65.2	72.7	60.0	62.5	62.5	77.8	63.3	75	50.5
–De Facto	34.6	21.7	18.2	30.0	25	25	11.1	27.3	12.5	37.5
–Separated	0	8.7	0	10.0	0	12.5	0	9.1	0	12.5
–Single	7.7	4.3	9.1	0	12.5	0	11.1	0	12.5	0
T1 Birth location (%)										
–Australia	73.1	82.6	81.8	80.0	87.5	100	77.8	72.2	75	87.5
–Other	26.9	17.4	18.2	20.0	12.5	0	22.2	27.8	25	12.5
T1 Income (%)										
–Up to $ 20,000	0	4.5	0	10.0	0	12.5	0	9.1	0	12.5
–$ 20,001–$ 40,000	8.0	22.7	9.1	20.0	12.5	12.5	11.1	18.2	12.5	25
–$ 40,001–$ 60,000	20.0	13.6	9.1	10.0	0	0	11.1	9.1	0	0
–$ 60,001–$ 80,000	28.0	27.3	36.4	20.0	37.5	25	33.3	27.3	37.5	25
–> $ 80,001	32.0	31.8	36.4	40.0	37.5	50	33.3	36.4	37.5	37.5
–Do not wish to divulge	12.0	0	9.1	0	12.5	0	11.1	0	12.5	0
T1 Highest level of education (%)										
–Did not finish school	3.8	12.0	0	0	0	0	0	0	0	0
–High School	7.7	24.0	0	27.3	0	25	0	25	0	25
–Certificate Level/Apprenticeship	23.1	4.0	9.1	9.1	12.5	12.5	9.1	8.3	10	12.5
–Advanced Diploma	19.2	4.0	36.4	0	25	0	36.4	0	30	0
–Bachelor degree	11.5	24.0	0	18.2	0	25	0	16.7	0	25
–Graduate diploma/certificate	19.2	16.0	36.4	27.3	37.5	25	36.4	25.0	40	25
–Postgraduate Degree	15.4	16.0	18.2	18.2	25	12.5	18.2	25.0	20	12.5

CBT, Cognitive Behavioral Therapy (intervention); TAU, Treatment as Usual (control); SD, Standard Deviation; T1, At randomization; T2, 9 weeks post-randomization; BDI-II, Beck Depression Inventory—second edition; BAI, Beck Anxiety Inventory.

(difference = −0.028%, 95%CI = −0.035 to −0.022). Although 68% of the promoter-associated *NR3C1* probes were less methylated in the CBT group, mean DNA methylation of all *NR3C1* promoter-associated probes did not differ significantly between the CBT and TAU groups (difference = 0.002%, 95% CI = −0.010 to 0.011) (61).

Brain Morphometry and White Matter Microstucture

Children from the CBT group had a thicker right lateral occipital cortex (difference = 0.13 mm, 95%CI = 0.005 to 0.26) and lingual gyrus (difference = 0.18 mm, 95%CI = 0.01 to 0.34). In the CBT group, Voxel-Based Morphometry analysis identified one cluster showing increased gray matter concentration in the right medial temporal lobe at p <0.05 uncorrected, and fixel-based analysis revealed reduced fiber-bundle cross-section in the Fornix, the Optical Tract, and the Stria Terminalis at p <0.01 uncorrected (62).

Behavior and Cognition

We found no significant differences in full scale IQ (difference: −3.2 IQ points, 95%CI = −16.1 to 9.7) or Total Problems Score (difference: −1.7 points, 95%CI = −30.5 to 27.1) between children in the CBT and TAU groups (57).

DISCUSSION

In this perspective article, we present a summary of findings from a longitudinal study based on a pilot RCT investigating the effects of

CBT during pregnancy for depression and anxiety on maternal mood, child behavior and cognition, and neurobiological outcomes including brain morphology and DNA methylation in children at a mean age of 5 years. Results from the DNA methylation analysis revealed no robust widespread DNA methylation differences between the CBT and the TAU group (61). However, the top 1,000 mostly differentially methylated CpG sites between the CBT group and TAU group showed overall lower average methylation in the CBT group. Among all candidate genes, an overall lower average methylation level was observed in the CBT group as well, which was not surprising regarding the overall lower wide-spread DNA methylation levels in the CBT group. Concurrently, the majority of promoter-associated probes across *NR3C1* showed lower average methylation in the CBT group compared to the TAU group. These results point to a possible beneficial effect of treatment of depression during pregnancy on DNA methylation overall and on candidate genes that have been associated with prenatal exposure to maternal stress, depression or anxiety, specifically, on the promoter region of *NR3C1*. In earlier studies, DNA methylation of the 1F region on the promoter of *NR3C1* showed increased methylation in children who were born to mothers who were depressed during their pregnancy (40, 63, 64). Increased methylation of CpG-rich areas in a promoter region of a gene, changes the activity of the DNA segment, typically by repressing its activity. Although Oberlander et al. did not examine whether DNA methylation of *NR3C1* was also associated with *NR3C1* gene expression, DNA methylation of *NR3C1* was on itself associated with increased stress responsiveness in 3-month-old infants (40). Dysregulation of HPA axis activity might be one of the basal mechanisms in developing certain mood disorders such as depression or anxiety. The fact that our study results indicate that CBT during pregnancy possibly decreases DNA methylation of the promoter region of *NR3C1*, is therefore promising.

The analyses of brain grey matter structure, volume and concentration and white matter connectivity yielded interesting results as well. We found that in medial and occipital brain regions, a number of voxels exhibited a trend towards a larger concentration of grey matter in the children from the CBT group compared to the TAU group (62). For the morphometric measures, we had a specific interest in the amygdala, as this structure is involved in emotional processing and stress-reactivity, and has been shown to be larger in children who were prenatally exposed to maternal depression. We hypothesized that in the CBT group smaller volumes of the amygdala would be observed, however, this was not the case. We also estimated and compared cortical thickness of regional gyri and sulci that have been shown to be thinner in children after prenatal depression or anxiety exposure. We found that in most brain regions, with some to a degree that statistical significance was reached, the cortex of the grey matter was thicker in the CBT compared to the TAU group, also after adjustment for relevant confounders. Finally, we also examined white matter tractography, by comparing the density and cross-section of white matter *fixels*. The most apparent finding was that children from the CBT group had smaller cross-section of white matter fiber bundles at a region which was correlated to the position and shape of the Fornix, the Stria Terminalis and Optical tract, which may indicate developmental differences of specific white matter microstructures involved in emotional and memory processing and the stress response.

Although both the results from the DNA methylation study as well as the majority of the results from the brain imaging study were pointing in the direction we had hypothesized, none of the results remained statistically significant after we considered the number of hypotheses tested. This did not come as a surprise however, given the very small numbers in the samples.

There were no robust differences in IQ scores between the CBT and TAU group. Also, we could not detect any differences in behavioral problem scores between both groups. In additional sub-analyses, we did observe that the severity of symptoms of depression or anxiety prior to allocation to either the CBT or the TAU group was associated strongly with lower scores on the cognitive scales, as well as increased scores of problematic behaviors (57). This indicated that the detrimental effects of depression and anxiety symptoms in pregnancy on clinical outcomes in children were still present, despite treatment. Women started treatment at a mean gestational age of around 19 weeks. Perhaps, an earlier intervention, during critical stages of embryonic/fetal brain development, would have led to stronger positive effects on neurodevelopmental outcomes in the children in later life, but this is highly speculative.

The lack of robust statistically significant findings may be attributable to a lack of power, as we were able to include only a small sample of children from the pilot RCT. Therefore, substantial larger studies are needed to investigate whether more subtle effects of CBT on cognition and behavior may occur. Also, attrition bias may have influenced the validity of our results, as there was some evidence that women who responded to the 5-year follow-up study had been less depressed or anxious at the start of treatment, compared to the overall original cohort, as well as the women that had responded to the 2-year follow-up. This may have been a contributive factor to the fact that the beneficial treatment effect that was evident at 9 months of age but less apparent at 2 years of age, was not present anymore at 5 years of age.

Evidently, we cannot establish whether the neurobiological trends associated with CBT that we observed in children at the age of 5 years, were actually the result of treatment effects on DNA methylation and brain development of the fetus *in utero*. It is likely that the positive effect of CBT on maternal mood seen after treatment and at 9 months postpartum has had an enduring effect on maternal mood and potentially on mother–child attachment and parenting skills postnatally, which on its turn may have affected DNA methylation profiles and brain development in the children in a positive manner. Nevertheless, in the follow-up studies at 2 years as well as 5 years postpartum, maternal symptoms of depression and anxiety were approximately similar in both the CBT and TAU group. However, as we did not assess parenting skills or included other variables that are indicative of maternal behavior and mother–child attachment postpartum, we cannot exclude the possibility that the trends that were seen in neurobiological outcomes were in fact mediated by the postnatal environment. Nonetheless, the opposite is also possible—the effects on DNA methylation and brain development may have been

stronger immediately after birth, and may have worn off in the subsequent years, which would explain why the effects observed were overall small and non-significant. Hence, this would also explain why at the age of 9 months' large treatment effects were observed on developmental outcomes, which could not be replicated at 2 and 5 years of age. However, the developmental assessments at 9 months and at 2 and 5 years old were not necessarily reflective of similar underlying developmental processes. It is possible that certain beneficial treatment effects on developmental outcomes were missed, simply because we did not measure them. Also, the positive effects of CBT in pregnancy on neurodevelopmental outcomes may only appear at a later age. Longer term assessment of the children to monitor the occurrence of psychopathology, and to measure cognition and behavior in, for example, early adulthood would therefore be highly informative.

A limitation of the study is that we were not able to address all potential biological mechanisms that may be involved in the association between prenatal exposure to maternal depression and adverse offspring neurodevelopment. For example, it would have been insightful if we had included maternal cortisol values during pregnancy, before and after treatment, as this would have provided further evidence whether maternal cortisol in pregnancy reflects the biochemical state of 'being depressed or not' and if this is potentially modifiable by psychological treatment. However, evidence from literature indicates that maternal depression in pregnancy is often not correlated with cortisol (31). Alternatively, maternal depression during pregnancy may increase DNA methylation of placental *HSD11B2*, which leads to a decrease in expression of the enzyme that deactivates active cortisol, thereby allowing more cortisol to cross the utero-placental barrier and reach the fetal circulation. However, evidence in humans supporting this is limited, at least for depression alone (65, 66). Also, the immune system has been shown to interact with cortisol metabolism, and (DNA methylation and gene expression of) indices of the immune system function such as lymphocytes have shown to act as mediating and/or moderating factors in the associations between maternal depression in pregnancy and altered child neurodevelopment (37, 67). Therefore, it would have been valuable if we had been able to analyze maternal blood samples as well as neonatal blood samples and/or placental samples for inflammatory as well as neuroendocrine parameters, besides DNA methylation statuses of candidate genes of for example, leukocytes. The reason these measures were not included in the original study design was because the main purpose of the original study was to assess the effects of CBT on depressive symptoms during pregnancy on the mothers clinically and whether this also translated into more beneficial infant development on the short-term. The study protocol that focused on neurobiological underlying processes 5 year postpartum, providing the main results that are included in this article, was designed 5 years' post-partum, and therefore we were not able to include these measures, which in retrospect, would have been highly informative.

Also, we did not include cortisol as an infant outcome measure at the follow-up at 5 years of age, as indicator of HPA axis functioning in the children after prenatal CBT treatment. Evidence from literature indicates that a single baseline cortisol value does not fully reflect HPA axis functioning, due to its wide variation across the day. Multiple values across a day-span are preferred, to consider the physiological variation in diurnal rhythm of the HPA-axis. Moreover, cortisol reactivity measures in response to a stressor may possibly predict later life health even better (68). Therefore, measuring cortisol would have been of much added value in our sample, had we performed a stress test with the children as well. However, research has shown that many stressors do not actually elicit a measurable HPA stress response in children. An extensive protocol such as the well-established Trier Social Stress Test (TSST) would potentially have elicited a robust stress response (69), but the children from our cohort were around 5 years old and too young to undergo the TSST. Also, the duration of the assessment procedure was around 3 to 4 h already, after which the children were generally very tired, and including a stress assessment would probably have led to resistance in the children, or for the parents to refuse overall participation. However, in accordance with the small but promising positive treatment effects that were seen on DNA methylation of the promoter region of the glucocorticoid receptor gene, it would be very interesting to re-invite the children in adolescence, to participate in a stress paradigm and measure concurrent cortisol values. A larger RCT (N = 230) on the effect of antenatal CBT on maternal depression and anxiety during pregnancy and child neurodevelopment at age 2 is currently running. An add-on study that aims to include neonatal (blood spot/buccal swabs) and child neurobiological (cortisol in saliva) measures is currently in progress.

CONCLUSION

Our findings indicate that CBT for antenatal depression may improve neurobiological outcomes in children, defined by decreased DNA methylation of genes involved in stress regulation and morphological brain changes in cortical areas associated with cognition, and white matter tracts involved in the stress response. However, results did not survive correction for multiple testing, and we could not confirm any subsequent beneficial effects on behavior and cognition (yet). A small sample size and possible attrition may have contributed substantially to the lack of robust findings, and larger studies are needed to make conclusive statements on whether CBT during pregnancy may positively affect neurobiological and neurodevelopmental outcomes in the offspring. Nevertheless, the current study is, to the best of our knowledge, the first to explore neurobiological outcomes in children in an RCT assessing a psychological intervention for maternal depression in pregnancy, and our results point to a possible positive effect on DNA methylation and brain development, which justifies further research in larger trials. A large RCT (N = 230) aiming to test the effect of antenatal CBT for depression and anxiety on child neurodevelopment is currently ongoing.

ETHICS STATEMENT

This study was approved by the Human Research Ethics Committees of Northern Health, Austin Health, and Mercy Health, Melbourne, Australia who approved the RCT (Trial Registration ACTRN12607000397415) and both follow-up studies. All

participants provided written informed consent prior to enrolment in the study.

AUTHOR CONTRIBUTIONS

The original RCT, with infant follow-up up to 24 months was designed and performed by JM, AG and CH. Study design of the 5-year follow-up was originally designed by SR, HB, and LB. JM, AG and CH contributed in the process of ethic approval. LB performed the data collection. RS, AS-O, AC and DP performed data analysis together with LB on MRI and epigenetic data, AG and LB analyzed neuropsychological data. All authors greatly contributed to refining study designs and methods, and reviewing of the manuscripts, which were written by LB. All authors agree to be accountable for the content of the work.

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Associations Between Child Maltreatment, Autonomic Regulation, and Adverse Cardiovascular Outcome in an Urban Population: The HELIUS Study

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Introduction: A mounting body of literature emphasizes the potential negative effects of adverse childhood experiences (ACEs) on both mental and physical health throughout life, including an increased risk for developing cardiovascular disease (CVD). Since CVD is one of the leading causes of mortality and morbidity worldwide, it is of great importance to advance our understanding of the effects of on CVD. This holds both for the actual incidence and for intermediate biological pathways that may convey CVD risk, such as imbalance in autonomic nervous system regulation, resulting in a chronically heightened sympathetic activity and lowered reactivity. In a large urban, multi-ethnic population-based cohort study we investigated whether there is an association between child maltreatment, CVD incidence and autonomic regulation.

Methods: Within the Health in an Urban Setting (HELIUS) study, a large, multi-ethnic population cohort study including n = 22,165 Amsterdam residents, we used logistic regression analyses to investigate the association between the number of self-reported types of child maltreatment (range 0–4), and self-reported adverse cardiovascular outcome (aCVO). Self-reported child maltreatment included emotional neglect, emotional abuse, physical abuse, and sexual abuse. Furthermore, in a subsample (n = 10,260), mean age 44.3, we investigated associations between child maltreatment, autonomic regulation, and aCVO using linear regression analyses. Both baroreflex sensitivity (BRS) and heart rate variability (HRV) were assessed as non-invasive indices of autonomic regulation.

Results: The number of endorsed child maltreatment types was significantly associated with a higher aCVO risk. The association remained significant after adjustment for demographic, socioeconomic, health-behavioral, and psychological covariates (p = 0.011,

odds ratio: 1.078, confidence interval: 1.018–1.142). The cumulative exposure to child maltreatment was negatively associated with BRS and HRV, but the association was no longer significant after correction for socioeconomic and demographic covariates.

Conclusion: In a large, multi-ethnic urban-population cohort study we observed a positive association between number of endorsed child maltreatment types and self-reported aCVO but not autonomic regulation, over and above the effect of relevant demographic, health, and psychological factors. Future studies should examine the potential role of the dynamics of autonomic dysregulation as potential underlying biological pathways in the association between ACEs and CVD, as this could eventually facilitate the development of preventive and therapeutic strategies for CVD.

Keywords: adverse childhood experience, cardiovascular disease, autonomic regulation, heart rate variability, baroreflex sensitivity

INTRODUCTION

According to the World Health Organization (WHO), the widely used umbrella term adverse childhood experiences (ACEs) refers to some of the most intensive and frequently occurring sources of stress that happens to someone before the age of 18 that the person remembers as an adult (1), either directly (e.g., abuse and neglect), or indirectly through their living environments (e.g., parental conflict, substance abuse, or mental illness). ACEs are common and affect a substantial part of our society. Estimations from multiple studies on the prevalence of ACEs in large healthy adult populations in Western countries vary from 34 to 62% of the population having experienced one or more ACEs (2–4).

ACEs, i.e. prolonged and/or severe (traumatic) stress experiences in childhood, can have lifelong consequences for a person's health and well-being (5, 6). Cross-sectional and longitudinal studies have previously observed associations between ACEs and subsequent altered functioning of a myriad of biological systems, including the nervous, endocrine, and immune system (7, 8). This may result in impaired emotional, cognitive, physical, and social functioning, and thereby affect mental and physical health. Additionally, ACEs have been described to indirectly negatively impact mental and physical health through associations with increased maladaptive lifestyle and behavior (such as smoking, alcohol use, physical inactivity, and an unhealthy diet) (5, 9–14); decreased access to life opportunities; and lack of economic stability, although the directionality of causality in these associations is difficult to establish (15). Thus, given the evidence of their negative long-term impact on public health, ACEs are of great concern.

One of the unfavorable health outcomes associated with ACEs is an increased risk for developing cardiovascular disease (CVD), one of the leading causes of mortality and morbidity worldwide (16). ACEs have been associated with a significantly increased risk of both self-reported and objectively verified CVD in several studies, including general population cohorts and CVD case–control studies [for systematic review see (17)].

The pathophysiological pathway through which ACEs increase the risk of CVD is likely to be multifactorial, with autonomic nervous dysregulation as one of the potential underlying physiological mechanisms leading to CVD risk (7). Both baroreflex sensitivity (BRS) and heart rate variability (HRV) are non-invasive indices of autonomic regulation. HRV, the beat-to-beat variation in the heartrate over time, is a reliable index of autonomic nervous regulation (18). Decreased HRV indicates an inability to reduce sympathetic activation of the heart (19). This makes the heart vulnerable to arrhythmia and sudden death and also accelerates development of atherosclerotic coronary artery disease (20, 21). Reduced HRV has been established as a prognostic risk factor for CVD. For example, lower HRV has been shown to be an independent predictor of cardiac events and mortality in both healthy individuals and patients with a history of myocardial infarction (19, 22). Additionally, BRS is the magnitude of change in heart rate interval in response to a change in systolic blood pressure (SBP) (17). The baroreflex is important for hemodynamic stability and for cardio protection. It has however repeatedly been demonstrated that a decrease in BRS is a prognostic factor for CVD and arrhythmic events post-myocardial infarction [MI (19)].

Current models assume that ACEs increase CVD risk by altering long-term patterns of autonomic regulation by disturbing the sympathetic and parasympathetic balance (7, 23), characterized by enduring higher sympathetic activity and lowered parasympathetic activity. Multiple studies have shown associations between ACEs and HRV, generally finding lower HRV to be associated with ACEs, but not all findings in non-clinical, depressed and adolescent samples have been consistent (24–28). The association between ACEs and BRS has not been examined before.

In this study, we aimed to investigate whether exposure to child maltreatment increases the risk for CVD, i.e. self-reported adverse cardiovascular outcomes (aCVO), and autonomic regulation (as measured with HRV and BRS) in the general adult population. The focus of the current study is on child maltreatment as this concerns a relatively common and directly experienced type of ACE, which may occur in the form of physical, sexual, and psychological abuse, and emotional

neglect, and has been previously observed to have pervasive consequences for subsequent health and well-being of affected individuals throughout life. We used data from the Health in an Urban Setting (HELIUS) study, which is a large representative urban cohort study, N = 22,615, among several ethnic groups living in Amsterdam, the Netherlands. The design of the HELIUS study guarantees a heterogeneous cross-section of the general Western population, generalizable on demographic factors such as socioeconomic status (SES), educational level, and ethnicity (29, 30).

METHODS

The HELIUS study is a large cohort study carried out in Amsterdam, the Netherlands. The HELIUS study mainly focuses on three disease categories: CVD, mental health, and infectious diseases. Between 2011 and 2015, baseline HELIUS data were collected among Amsterdam residents of South-Asian Surinamese, African Surinamese, Turkish, Moroccan, Ghanaian, and Dutch origin. Persons were randomly sampled, by ethnic origin, *via* the municipality register of Amsterdam and received an invitation to take part in the study. Contact could be made with 55% of selected invitees, of which approximately 50% responded and agreed to participate, leading to a total response rate of 28%. Previous non-response analyses revealed only minor differences in socioeconomic characteristics between invited individuals agreeing to participate, invited individuals not participating, and non-invited eligible individuals, indicating that the HELIUS cohort is representative for the investigated ethnic groups living in Amsterdam [for details see (29, 30). Data were collected through an extensive questionnaire and a physical examination that included the collection of biological samples. The Institutional Review Board of the Amsterdam UMC location AMC approved the study's protocols and all participants gave written informed consent. For the current analyses, we used data of n = 22,165 participants for whom data from the questionnaire regarding child maltreatment and aCVO as well as the physical examination were available.

Measures

Child Maltreatment

The occurrence of child maltreatment was assessed using a questionnaire based on the NEMESIS Trauma questionnaire (31). It contains four items, reflecting specific types of maltreatment experienced before the age of 16: emotional neglect (ignored or unsupported), physical abuse (kicked, hit, bitten, or hurt), emotional abuse (yelled at, insulted, or threatened), and sexual abuse (any unwanted sexual experience). A Likert scale was used to answer these questions (never–once–sometimes–often–would rather not say). Maltreatment types were scored as endorsed when experienced sometimes or often, except for sexual abuse which was scored as endorsed when experienced at least once. Finally, the total number of maltreatment types endorsed was calculated (range: 0–4) (see below for details on imputation strategy in case of missing data).

aCVO

History of CVD, i.e. aCVO, was based on three self-report items in the HELIUS questionnaire: 1) Have you ever had a heart attack (MI)? 2) Have you ever had a stroke? 3) Have you ever had a dotter procedure (angioplasty) or a bypass operation on your heart or legs? The scale was dichotomized to 0 (no reported CVD) and 1 (at least one item endorsed). Three hundred seventeen participants were excluded due to missing data on self-reported CVD.

Autonomic Regulation

Electrocardiogram (ECG) measurements were performed in all participants (MAC 1600 System, GE Healthcare). Due to logistic constraints, only a subset of n = 13,726 participants was subjected to continuous blood pressure (BP) measurement by finger plethysmography using the Nexfin device (BMEYE, Amsterdam). BP measurements were obtained for 3–5 min in the supine position after 10-min rest. Automated analysis of the hemodynamic data was performed using custom-written software in Matlab (R2018b, The MathWorks, Inc. Natick) as described elsewhere (32). In brief, to remove noise and possible ectopic beats, the beat-to-beat dataset was filtered using a local moving median filter with a length of nine beats. Beats which duration diverted more than 25% of the mean interbeat interval (IBI), the duration between successive heartbeats, from the local median IBI were removed. Participants with no detected sinus rhythm on the ECG or more than 20% removed beats were excluded (32). This yielded n = 10,260 participants with available HRV and BRS data. We observed several small but statistically significant differences between included and non-included participants (see **Supplementary Table 1**). Compared to non-included participants, those included were more commonly males (45.8% versus 39.1%) and on average 5 months younger. Also, included participants had a somewhat higher BMI, but reported a lower number of maltreatment types experienced and lower prevalence of CVD.

HRV

HRV was determined by calculation of the mean IBI, the square root of the mean squared successive differences between adjacent normal-to-normal (NN) intervals (RMSSD) and the standard deviation of NN intervals (SDNN), following international guidelines (33). For the analyses, both RMSSD and SDNN (in ms) were included, as they both measure different aspects of HRV (34, 35). RMSSD mostly reflects the parasympathetic system, while SDNN reflects all the cyclic components responsible for HRV in the period of recording with the influence of the sympathetic system and all other influential systems on the heart rate included (33, 35, 36).

BRS

Estimates of BRS were obtained from beat-to-beat changes in SBP and heart rate, using the xBRS method. First, the change in heart rate in response to change in BP was assessed, using a cross-correlation method over a sliding 10-s window with various delay compensations (37, 38). Second, for each segment with a significant positive cross-correlation, xBRS was

obtained by dividing the standard deviation of the IBI by the standard deviation of the SBP. The overall xBRS (in ms/mm Hg) is the geometric mean of all obtained xBRS values per segment with a significant positive cross-correlation.

Covariates

Socioeconomic and demographic variables included were age, sex, ethnicity, and educational level. Ethnicity was divided into seven groups: Dutch, South-Asian Surinamese, African Surinamese, Ghanaian, Turkish, Moroccan, and "other." This latter group consists of participants with a Javanese-Surinamese origin, other/unknown Surinamese origin, or other/unknown ethnic origin, which were combined due to relatively small sample size (2.5% of total sample size). In all analyses, ethnic minority groups were compared to the Dutch ethnicity. Educational level was divided into two categories: 1) no or lower education (no schooling, elementary schooling only, lower vocational schooling, or lower secondary schooling) and 2) intermediate or higher education (intermediate vocational schooling, intermediate/higher secondary schooling, higher vocational schooling, or university) (30).

Furthermore, the following health behaviors and anthropometric measurements were taken into account: current smoking (yes/no), alcohol intake in past 12 months (yes/no), physical activity (reaching the international goal of 30 min per day on at least 5 days per week: yes/no), and body mass index (BMI) (measured during physical examination, kg/m^2), as well as current stress (negative life events in the last 12 months: yes or no). A broad range of studies have consistently shown associations between ACEs and higher risk of adult smoking (12), obesity (15), physical inactivity (39) and heavy alcohol use (5), and increased experienced stressors in adulthood, as well as associations between these variables and aCVO risk, and thus these factors represent potential mediating pathways in the child maltreatment–aCVO association.

Because of the expected influence of use of antihypertensive medication on both HRV and BRS, use of antihypertensives (dichotomous) was also corrected for in analyses including autonomic measures.

Statistical Analysis

For the data analyses in this study, IBM SPSS statistics version 25.0 was used. A two-sided p-value of <0.05 was considered significant. Because of the high percentage of missing values in child maltreatment data (range: 4.4% for Dutch participants, up to 12% for Ghanaian participants), multiple imputation was performed to avoid underestimation of the prevalence of child maltreatment and thereby biased results on the probable association between child maltreatment and our outcomes. Multiple imputation is an effective method for dealing with missing data (40), and is becoming increasingly common, also specifically for studies on ACEs (41, 42). As missingness (item either left blank or scored "would rather not say") correlated with the large majority of variables, the missing data for the four child maltreatment items were imputed in SPSS using Markov Chain Monte Carlo imputation with fully conditional specification with auxiliary variables (main effects and two-way interactions among categorical predictors) and predictive mean matching. All variables used for our final analysis were included as auxiliary variables (40). Eventually 30 imputations were needed to obtain adequate imputation efficiency. All reported findings concern the pooled results from these 30 imputed datasets.

We first investigated whether child maltreatment were significantly associated with aCVO, using binomial logistic regression analyses. Secondly, we investigated whether child maltreatment were significantly associated with the three autonomic measures: RMSSD, SDNN, and BRS, using three separate sets of linear regression analyses. Third, we investigated whether the three autonomic measures were associated with aCVO, using three separate sets of binomial logistic regression analyses. Prior to each regression analysis, it was confirmed that relevant statistical assumptions were met.

In each regression analysis, adjustment for covariates was performed in a stepwise manner. First, we assessed a model without any covariates added. Second, we added a block of covariates regarding demographic and socioeconomic variables that may potentially confound the association between child maltreatment and CVD. Third, a block of covariates regarding health-behavioral characteristics and chronic stress was added. This latter block represents potential mediating factors in the child maltreatment–aCVO association. It was included in the regression models as we aimed to examine whether there was an additional effect of child maltreatment on CVD over and above these previously described mediating pathways. Additionally, for the models containing autonomic measures only, antihypertensive medication use added as additional covariate in a separate block, before adding the other covariate blocks. Furthermore, a sensitivity analysis was performed to adjust for the potential influential effect of already experienced aCVO on the relationship between child maltreatment and autonomic regulation, excluding all participants reporting aCVO.

To assess whether moderation effects between ACEs and age or gender should be considered in addition to their main effects, regression analyses on associations between child maltreatment and aCVO, HRV, and BRS were additionally performed including the interaction effects between number of endorsed child maltreatment types and gender or age group respectively. For this purpose, the age variable was categorized into four categories containing equal percentages of participants (i.e. quartiles): 18–32, 33–45, 46–53, and 54–70. The first three categories were compared to the eldest category, because aCVO was expected to be highest within that group. Sex and age did not significantly moderate the effect of child maltreatment on BRS or HRV, nor the effect of child maltreatment on aCVO (**Supplementary Tables 5A, B**), and therefore we considered only main effects of age and gender in the results.

RESULTS

Sample characteristics per ethnic group are shown in **Table 1**. The average age of all participants was 44.3 (SD = 13.2) years, with 57.8% women. Overall, 7676 participants (34.6%)

experienced any type of child maltreatment, and 1135 participants (5.2%) reported a history of aCVO.

Association Between Child Maltreatment and aCVO

A higher number of endorsed ACE types was significantly associated with a 1.1 times higher risk for aCVO, as shown in **Table 2**. After correction for all covariates, this association remained significant, with each additional child maltreatment type endorsed increasing the odds of aCVO by 7.8% [95% confidence interval (CI): 1.05–1.17, p = 0.011]. The corresponding table is shown in **Supplementary Table 2**.

Association Between Child Maltreatment and Autonomic Regulation

In the models without covariates, the number of endorsed ACE types was significantly associated with RMSSD and BRS, but not SDNN (**Table 3**). An increasing number of child maltreatment types endorsed was significantly associated with lower BRS and lower RMSSD. These associations were not affected by additionally adjusting for antihypertensive medication use but were no longer present after adding the sociodemographic covariates to the model (see **Supplementary Tables 3A, B**). This was not affected by the final step of adding the covariates concerning health-behavioral characteristics and current stress

TABLE 1 | Baseline characteristics of the study population.

	Total (N = 22,165)	Dutch (N = 4564)	South-Asian Surinamese (N = 3043)	African Surinamese (N = 4151)	Ghanaian (N = 2339)	Turkish (N = 3614)	Maroccan (N = 3906)	Other (N = 548)
Sociodemographics								
Age	44.3 ± 13.2	46.2 ± 14.1	45.5 ± 13.4	47.9 ± 12.5	44.7 ± 11.2	40.4 ± 12.2	40.5 ± 12.9	47.5 ± 12.5
Female	12,810 (57.8%)	2475 (54.2%)	1672 (54.9%)	2535 (61.1%)	1434 (61.3%)	1980 (54.8%)	2392 (61.2%)	322 (58.8%)
Educational level								
No–lower	9679 (44.1%)	796 (17.5%)	1447 (47.8%)	1708 (41.5%)	1577 (68.7%)	2024 (56.6%)	1899 (49.1%)	228 (42.5%)
intermediate–higher	12,279 (55.9%)	3743 (82.5%)	1580 (52.2%)	2407 (58.5%)	720 (31.3%)	1552 (43.4%)	1969 (50.9%)	308 (57.5%)
Health behavior								
Smoking	5302 (24.0%)	1129 (24.8%)	861 (28.4%)	1309 (31.7%)	104 (4.5%)	1240 (34.6%)	525 (13.5%)	134 (24.8%)
Drinking alcohol	11,221 (50.9%)	4151 (91.1%)	1708 (56.4%)	2826 (68.6%)	1101 (47.6%)	813 (22.7%)	286 (7.4%)	336 (62.6%)
BMI	27.1 ± 5.3	24.8 ± 4.2	26.3 ± 4.8	27.8 ± 5.5	28.4 ± 5.0	28.6 ± 5.7	27.6 ± 5.2	26.7 ± 5.0
Child maltreatment								
Any type	7676 (34.6%)	1689 (37.0%)	1132 (37.2%)	1645 (39.6%)	688 (29.4%)	1231 (34.1%)	1066 (27.3%)	225 (41.1%)
Emotional neglect	5542 (25.0%)	1287 (28.2%)	828 (27.2%)	1070 (25.8%)	412 (17.6%)	1017 (28.1%)	763 (19.5%)	165 (30.1%)
Emotional abuse	3615 (16.3%)	734 (16.1%)	609 (20.0%)	820 (19.8%)	278 (11.9%)	514 (14.2%)	538 (13.8%)	122 (22.3%)
Physical abuse	3640 (16.4%)	443 (9.7%)	626 (20.6%)	969 (23.3%)	427 (18.3%)	505 (14.0%)	556 (14.2%)	114 (20.8%)
Sexual abuse	1871 (8.4%)	531 (11.6%)	232 (7.6%)	585 (14.1%)	156 (6.7%)	116 (3.2%)	181 (4.6%)	70 (12.8%)
Number of types experienced								
0	14,489 (65.4%)	2875 (63.0%)	1911 (62.8%)	2506 (60.4%)	1651 (70.6%)	2383 (65.9%)	2840 (72.7%)	323 (58.9%)
1	3464 (15.6%)	856 (18.8%)	455 (15.0%)	644 (15.5%)	333 (14.3%)	628 (17.4%)	461 (11.8%)	87 (15.9%)
2	2000 (9%)	455 (9.9%)	279 (9.2%)	408 (9.8%)	169 (7.2%)	327 (9.0%)	304 (7.8%)	58 (10.6%)
3	1640 (7.4%)	282 (6.2%)	307 (10.1%)	389 (9.4%)	141 (6.0%)	233 (6.5%)	236 (6.0%)	52 (9.5%)
4	572 (2.6%)	96 (2.1%)	90 (2.9%)	204 (4.9%)	45 (1.9%)	43 (1.2%)	65 (1.7%)	28 (5.1%)
CVD	1135 (5.2%)	167 (3.7%)	271 (9%)	216 (5.3%)	101 (4.5%)	233 (6.6%)	126 (3.3%)	21 (3.9%)
Autonomic regulation								
N	10,260	2045	1341	2079	1251	1729	1815	0
BRS	13.43 ± 9.07	14.98 ± 10.55	12.51 ± 8.31	12.96 ± 8.44	12.63 ± 8.82	12.69 ± 8.39	14.16 ± 9.61	–
RMSSD	46.73 ± 29.46	49.70 ± 32.43	43.63 ± 30.09	46.68 ± 29.60	47.35 ± 26.97	44.13 ± 27.26	47.77 ± 28.7	–
SDNN	52.41 ± 27.82	58.50 ± 31.16	51.43 ± 28.97	50.73 ± 26.84	49.63 ± 26.75	50.71 ± 26.10	51.72 ± 25.31	–

Baseline characteristics per ethnic group, described as means and standard deviations for continuous variables, and frequency and percentage for categorical variables. CVD, cardiovascular disease; BRS, baroreflex sensitivity; RMSSD, a parameter reflecting heart rate variability calculated as the square root of the mean squared successive differences between adjacent normal-to-normal interbeat intervals; SDNN, a parameter reflecting heart rate variability calculated as the standard deviation of normal-to-normal interbeat intervals; BMI, body mass index.

TABLE 2 | Logistic regression analyses on the association between child maltreatment and adverse cardiovascular outcome (aCVO).

	R^2	β	Odds ratio	95% Confidence interval	p
Model 1	0.002	0.101	1.106	[1.049, 1.167]	<0.001
Model 2*	0.150	0.108	1.114	[1.054, 1.178]	<0.001
Model 3*†	0.159	0.096	1.100	[1.039, 1.165]	0.001
Model 4*†‡	0.164	0.075	1.078	[1.018, 1.142]	0.011

Each model shows the regression results of child maltreatment–number of types endorsed.
**Adjusted for sociodemographic covariates (sex, age, education, and ethnicity).*
†Adjusted for health-behavioral covariates (smoking, alcohol, body mass index, physical activity).
‡Adjusted for psychological covariate (current stress).

TABLE 3 | Multiple linear regression analyses on association between child maltreatment and xBRS and heart rate variability.

	R^2	β	95% Confidence Interval	p
xBRS				
Model 1	0.001	–0.016	[–0.028, –0.005]	0.006
Model 2*	0.068	–0.017	[–0.028, –0.006]	0.003
Model 3*†	0.370	–0.002	[–0.009, 0.010]	0.907
Model 4*†‡	0.395	–0.001	[–0.007, 0.008]	0.981
RMSSD				
Model 1	0.001	–0.015	[–0.025, –0.004]	0.007
Model 2*	0.031	–0.015	[–0.026, –0.005]	0.004
Model 3*†	0.220	–0.003	[–0.013, 0.006]	0.530
Model 4*†‡	0.233	–0.007	[–0.016, 0.003]	0.169
SDNN				
Model 1	0.000	–0.006	[–0.014,0.003]	0.196
Model 2*	0.041	–0.006	[–0.015,0.002]	0.148
Model 3†	0.184	–0.003	[–0.005,–0.011]	0.760
Model 4*†‡	0.196	0.001	[–0.007, 0.009]	0.747

N = 10,260. Each model shows the regression results of child maltreatment-number of types endorsed on xBRS/RMSSD and SDNN.
**Adjusted for antihypertensive medication.*
†Adjusted for sociodemographic covariates (sex, age, education, and ethnicity).
‡Adjusted for health-behavioral and psychological covariates (smoking, alcohol, body mass index, physical activity, current stress).
xBRS, baroreflex sensitivity; RMSSD, a parameter reflecting heart rate variability calculated as the square root of the mean squared successive differences between adjacent normal-to-normal interbeat intervals; SDNN, a parameter reflecting heart rate variability calculated as the standard deviation of normal-to-normal interbeat intervals.

to the model. Results remained unchanged after excluding participants reporting previous aCVO (**Supplementary Table 3C**).

Associations Between Autonomic Regulation and aCVO

The main effects of BRS, RMSSD, SDNN, on aCVO were all significant, also after adjustment for antihypertensive medication use. Lower BRS, RMSSD, and SDNN were associated with increased odds for aCVO (**Table 4**). For both HRV parameters, the association was no longer significant after adding socioeconomic covariates to the model (see **Supplementary Table 4B**). For BRS, the association remained significant after adjusting for sociodemographic covariates, but was no longer significant after adding the additional covariates concerning health-behavioral characteristics and current stress (see **Supplementary Table 4A**).

DISCUSSION

In this large, population-based, multi-ethnic urban cohort study, we tested whether exposure to child maltreatment as a specific form of direct ACEs is associated with higher risk of self-reported history of CVD and autonomic regulation as CVD risk factor. This is to our knowledge, the first study on the association between child maltreatment and CVD risk in a representative urban population: heterogeneous and generalizable on demographic factors such as SES, educational level, and ethnicity (29, 30).

Our study confirmed that child maltreatment is significantly associated with higher risk for CVD later in life. This association remained significant after adjusting for potentially relevant covariates. With every additional child maltreatment type reported, the odds for reporting aCVO was 7.8% (95% CI: 1.018–1.142) higher. Thus, child maltreatment was associated with an increased risk for adult aCVO over and above the effects of a range of sociodemographic, health-behavioral, and current stress factors, which were previously found to be associated with both increased self-reported ACEs and risk for CVD (3, 43, 44).

Our finding is in concordance with existing literature that ACEs are important determinants of health problems in adulthood and more specifically consistent with the results of previous studies examining the association between childhood maltreatment and CVD (39). These previous studies also reported a linear association between cumulative exposure and increased risk for self-reported

TABLE 4 | Logistic regression analyses on the association between adverse cardiovascular outcome (aCVO) and xBRS and heart rate variability.

	R^2	β	Odds Ratio	95% Confidence Interval	p
xBRS					
Model 1	0.041	−0.882	0.414	[0.356, 0.470]	<0.001
Model 2*	0.118	−0.534	0.586	[0.499, 0.689]	<0.001
Model 3*†	0.135	−0.209	0.811	[0.673, 0.978]	0.028
Model 4*†‡	0.192	−0.071	0.932	[0.769, 1.129]	0.470
RMSSD					
Model 1	0.018	−0.652	0.521	[0.441, 0.616]	<0.001
Model 2*	0.110	−0.360	0.698	[0.588, 0.828]	<0.001
Model 3*†	0.179	0.015	1.015	[0.844, 1.220]	0.877
Model 4*†‡	0.197	0.011	1.011	[0.838, 1.220]	0.909
SDNN					
Model 1	0.022	−0.868	0.420	[0.344, 0.513]	<0.001
Model 2*	0.111	−0.442	0.643	[0.524, 0.789]	<0.001
Model 3*†	0.179	−0.117	0.890	[0.717, 1.103]	0.286
Model 4*†‡	0.197	−0.080	0.923	[0.741, 1.150]	0.476

Each model shows the regression results of xBRS, RMSSD, and SDNN.

**Adjusted for antihypertensive medication.*

†Adjusted for sociodemographic covariates (sex, age, education, and ethnicity).

‡Adjusted for health-behavioral and psychosocial covariates (smoking, alcohol, body mass index, physical activity, current stress).

xBRS, baroreflex sensitivity; RMSSD, a parameter reflecting heart rate variability calculated as the square root of the mean squared successive differences between adjacent normal-to-normal interbeat intervals; SDNN, a parameter reflecting heart rate variability calculated as the standard deviation of normal-to-normal interbeat intervals.

CVD after adjustment for relevant covariates. However, this is the first study establishing this relationship in a heterogeneous, multi-ethnic, and thus Western urban population representative cohort as the HELIUS cohort.

In addition to self-reported history of CVD, we also examined the association between child maltreatment and autonomic regulation as CVD risk factor. We did find, as we expected, that the cumulative exposure to child maltreatment was negatively associated with autonomic regulation within models without any covariates added. However, this association disappeared after adding the sociodemographic covariates to the models, which in themselves were previously found to be associated with increased risk for ACEs and CVD (5, 12–16, 39). In agreement with our findings, some previous studies also did not find a significant direct association between HRV and ACEs. The study by van Ockenburg et al. (25), based on a randomly selected large cohort of people with albuminuria, found significantly lower HRV in individuals reporting ACEs, but as in the current study this association also disappeared after correcting for sociodemographic and health behavioral covariates (25). Winzeler et al. (24) found an association between ACEs and HRV in young healthy women when the HRV was measured during performance of a stress task and not during baseline measurements (24), which is in concordance with the null findings in our study, with HRV also measured during resting conditions.

Interestingly, to verify the assumed association between autonomic (dys) regulation and aCVO in our cohort, we also investigated associations between HRV, BRS, and aCVO. Contrary to our expectations, we observed that the initially observed negative associations between self-reported CVD and the objective measures of autonomic regulation were no longer present in the corrected models. Upon adding the potential sociodemographic confounders, the association between self-reported CVD and both HRV indices was no longer significant. Initially, we hypothesized that the absence of a stable association between HRV and CVD, which seems to be contradictory to the existing literature (19, 22), could be influenced by the composition of our cohort. First of all, as our cohort is relatively young and correspondingly aCVO prevalence is relatively low (5.2%), we may have had limited signal to detect these associations in the whole sample. However, we found that age was not a significant moderator in any of the associations. Secondly, the HELIUS study includes both individuals without prior CVD and individuals who have already experienced one or more CVD events, and thereby departs from the existing literature which investigated the association between autonomic regulation and future CVD events in either healthy populations or specific populations of CVD patients (19, 22, 45–53). However, a sensitivity analysis revealed that our results remained unchanged after excluding participants who reported CVD. The association between BRS and CVD remained significant upon adding the sociodemographic covariates, but was no longer significant upon additionally adding the health-behavioral and current stress factors, indicating that these factors may be mediating pathways influencing the formerly observed association between BRS and CVD (19).

Thus we did not observe an association between child maltreatment and autonomic regulation, nor an association between autonomic regulation and CVD after inclusion of covariates, although we cannot exclude that measuring these indices during specific physical or psychological challenges would reflect another pattern. Alternatively, ACEs including child maltreatment may trigger, besides ANS, a cascade of molecular alterations in other systems that regulate stress responses and may be involved in CVD development, such as neuroendocrine, immune systems, endothelial damage or accelerated atherosclerosis (54).

In addition, there may be psychological mechanisms that may also play a role in the association between ACEs, including child maltreatment, and increased CVD risk, such as maladaptive cognitive models, impaired attachment, dysfunctional coping behaviors, and unhealthy peer associations (55). Moreover, studies suggest that ACEs and especially child maltreatment could induce emotional problems, including depression, anxiety, and affective lability, and this could be associated with CVD risk in adulthood (26). Thus, poor mental health could either moderate or mediate associations between ACEs and health outcomes (56). Additionally, use of psychotropic medication may additionally influence these associations.

Furthermore, adversity is only one part of the equation regarding childhood environmental influences on future health. One could argue that in the face of adversity, neither disease nor resilience is a certain outcome. The presence of protective factors, particularly safe, stable, and nurturing relationships, can often mitigate the consequences of ACEs (57, 58). Also neighborhood greenness for example might buffer against the detrimental effects of stress by specifically promoting activity of the parasympathetic nervous system in restoring the body to a calm state after stress reactivity (59). However, these factors were not considered in the current study.

Strengths and Limitations

There are several strengths and weaknesses of this study that need to be considered when interpreting our results. The first major strength of this study is that the study was conducted in a large cohort, which provides notable statistical power. A random sampling technique was used through the municipality register of Amsterdam, which guarantees a non-selective, community recruited general population study sample. It is unlikely that the relatively low response rate of 28% has led to selection bias, as analysis of non-responders within our cohort established that there was no difference in socioeconomic characteristics between participants and non-participants (30).

Second, with the prevalence rate of any type of child maltreatment (around 30%) being in line with that of a large Dutch study on its prevalence in the general population (31), the experiences of participants in this study are representative for the Dutch general adult population, and results of our study are thus likely generalizable.

Potential limitations and weaknesses of our study also require consideration. First, the HELIUS data are cross-sectional, this poses constraints on the directionally in the investigated associations, especially in the association between autonomic regulation and CVD. Furthermore, the measurement of CVD by self-report may have led to under-reporting or over-reporting when compared to direct assessment of CVD events, although previous studies have shown a high degree of specificity for self-reported CVD and stroke (60, 61).

Moreover, we measured four types of child maltreatment: emotional neglect, psychological abuse, physical abuse, and sexual abuse, which certainly does not cover the full spectrum of ACEs. Furthermore, only the number of endorsed types of child maltreatment were assessed, not the overall severity, frequency of distinct types of maltreatment, or perceived impact of these experiences. We also did not examine the developmental timing and chronicity of maltreatment, which may also have an influence on the associations we investigated. Recent evidence indicates a distinctive impact of childhood adversity type and timing on physical and mental health and neurobiological correlates in adulthood, supporting the notion of stress-sensitive periods in (organ) development in childhood (62).

In addition, since the assessment of child maltreatment was based on retrospective self-report, effects of memory biases cannot be excluded. Moreover, child maltreatment may also occur before children have the cognitive ability to remember such events. Evaluating retrospective self-report to assess childhood maltreatment, a study performed on retrospective recalls of sexual and physical abuse, as well as physical and emotional neglect, ascertained retrospective surveys to be sufficiently valid (63). In contrast, a recent meta-analysis and systematic review performed on the extent of agreement between retrospective and prospective measures of child maltreatment concluded that prospective and retrospective measures cannot be used interchangeably to study risk mechanisms and associations with health outcomes (64). They mention that "caution should be used in assuming that retrospective reports accurately represent experiences, rather than perceptions, interpretations or existential recollections." Thus, ideally our study should be replicated in a prospective design.

Moreover, missing data in the child maltreatment questionnaire were significantly more frequent in every ethnicity compared to the Dutch ethnicity, which may be related to two factors. First, there could be a larger component of shame and taboo within some ethnicities to report these adverse experiences (65). Second, the ethnic differences may have resulted from the formulation of our items on child maltreatment: it is possible that some participants would have endorsed the objective explanation (e.g. being beaten during childhood), but did not agree on the following interpretation of that behavior as representative of maltreatment (e.g. having experienced physical abuse). However, we applied multiple imputation with auxiliary variables to deal with the impact of non-randomly missing data, which presumably resulted in a more accurate estimation of the relationship between maltreatment and our outcome variables (40).

Finally, although our analyses concerning BRS and HRV included over 10,000 participants, these analyses only included 46.3% of the sample. Due to logistic constraints concerning availability of equipment, continuous BP measurements were not performed for 28.1%. An additional 15.6% of participants were excluded from analyses upon data preprocessing. We observed several statistically albeit small significant differences between included and non-included participants, and therefore cannot exclude results were impacted by this selective subset. Also, BRS and HRV were only assessed once. As these measurements are highly dynamic within an individual (66), the assessment could be more valuable after repeated measurements. Moreover, HRV and BRS were only assessed in

resting conditions. We can therefore not exclude that associations between child maltreatment and the sympathovagal balance would be present under stressful conditions, such as in the study by Winzeler et al. (24).

Conclusion, Implications, and Future Directions

The use of this large, population urban population representative sample provides insight into the long-term correlates of child maltreatment. A positive association was established between cumulative ACEs in the form of child maltreatment and risk on aCVO, over and above the effect of relevant demographic, health, and psychological factors. The association between child maltreatment and autonomic regulation indices was no longer present after correcting for sociodemographic factors. The quality of research on this topic will be strengthened with prospective longitudinal studies starting in early age and continuing into old age, more expansive measurement of child maltreatment, and other ACE types as well as potential resiliency factors and direct and objective assessments of CVD events and assessment of dynamic autonomic regulation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The HELIUS study has been approved by the Ethical Review Board of the Academic Medical Center Amsterdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MB, MZ, and AL contributed to the conception and design of the current study. MS and DC organized the database. MB and MZ performed the statistical analysis. MB wrote the first draft of the manuscript. MZ and AL wrote sections of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

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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.2020.00069/full#supplementary-material

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Not the Root of the Problem—Hair Cortisol and Cortisone Do Not Mediate the Effect of Child Maltreatment on Body Mass Index

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Background: Experiencing maltreatment during childhood exerts substantial stress on the child and increases the risk for overweight and obesity later in life. The current study tests whether hair cortisol—a measure of chronic stress—and its metabolite cortisone mediate the relation between abuse and neglect on the one hand, and body mass index (BMI) on the other.

Method: The sample consisted of 249 participants aged 8 to 87 years (M = 36.13, SD = 19.33). We collected data on child abuse and neglect using questionnaires, measured cortisol and cortisone concentrations in hair, and BMI. In a structural model, the effects of abuse and neglect on hair cortisol, hair cortisone, and BMI were tested, as well as the covariance between hair cortisol and BMI, and hair cortisone and BMI.

Results: Within the sample, 23% were overweight but not obese and 14% were obese. Higher levels of experienced abuse were related to higher cortisone concentrations in hair ($\beta = 0.24$, $p < .001$) and higher BMI ($\beta = 0.17$, $p = .04$). Neglect was not related to hair cortisol, hair cortisone, or BMI. Hair cortisol and cortisone did not mediate the association between maltreatment, and BMI. Sensitivity analyses demonstrate the same pattern of results in a subsample of adult participants currently not living with their parents. However, in younger participants who were still living with their parents, the associations between abuse and cortisone ($\beta = 0.14$, $p = .35$) and abuse and BMI ($\beta = 0.02$, $p = .92$) were no longer significant.

Conclusion: These findings confirm that experiencing abuse is related to higher BMI but suggest that hair cortisol and cortisone are not the mechanism underlying the association between child maltreatment and BMI. This is the first study to show abuse may be

associated to elevated concentrations of hair cortisone—evidence of long-term alterations in chronic stress levels. Future research may benefit from exploring the effects of maltreatment on weight gain in longitudinal designs, including measures of other potential mediators such as eating as a coping mechanism, and more direct indicators of metabolic health.

Keywords: child maltreatment, abuse, neglect, hair cortisol, hair cortisone, hypothalamic–pituitary–adrenal axis, body mass index

INTRODUCTION

Abuse and neglect are adverse childhood experiences that exert substantial stress on the child, and violate expectations of a safe and stable environment (1, 2). Experiencing abuse or neglect during childhood increases the risk for a number of negative mental (3–5) and physical health outcomes (6–8)—among them an increased risk for overweight and obesity later in life (9–11). The relation between childhood maltreatment and overweight has been confirmed by two meta-analyses including 190,285 and 112,708 participants, respectively (12, 13). Longitudinal studies with several assessments have shown that maltreatment is related to accelerated weight gain (14–17), but report conflicting results with regard to which type of maltreatment drives this effect.

Overweight and obesity present a global health challenge with rising prevalence (18) and with increased risk for diabetes, cardiovascular disease, cancer, and overall mortality (19–24). Unfortunately, it has proven difficult to develop effective interventions to prevent obesity (25), but targeting the mechanisms that underlie the relation between maltreatment and weight gain, such as stress, might offer new effective strategies (26). It has been proposed that mental health mediates the association between maltreatment and BMI. One study investigated posttraumatic stress disorder as a potential mechanism but only found a weak mediation effect (27). Another study found that depression fully mediated the association between physical abuse and BMI in girls but not in boys and did not play a role for other types of maltreatment (17). An alternative approach would be to focus on biological factors such as stress physiology. It has been suggested that maltreatment experiences and increases in BMI could be connected through chronically elevated levels of cortisol (9, 12). Cortisol has been implicated both as a consequence of maltreatment and as a factor involved in weight gain. Therefore, the current study tests the mediational role of hair cortisol (and its metabolite cortisone) in the association between abuse and neglect on the one hand, and BMI on the other hand.

If abuse and neglect are encountered during developmentally sensitive periods, they have the potential to not only elicit an acute stress response but also to reprogram one of the central human stress response systems: the hypothalamic–pituitary–adrenal axis [HPA axis; (28)]. When confronted with a stressor the HPA axis is activated and after a number of intermediary steps, cortisol is released from the adrenal gland and prepares the body to respond to danger (29). Therefore, HPA axis activity is often indexed by measuring cortisol levels. One meta-analysis found evidence of blunted wake-up cortisol levels in maltreated children and adults, with stronger effect sizes in agency-referred samples, but no effect of maltreatment on the cortisol awakening response and the diurnal cortisol pattern (30). Another meta-analysis focused on studies measuring the cortisol response following a social stressor (31). There was evidence of blunted cortisol reactivity in maltreated individuals and this effect was stronger in adults than in children. This can be explained from a theoretical perspective: Both the allostatic load model and the attenuation hypothesis argue that stress will initially elicit an increased stress response in the form of heightened cortisol levels, but over a prolonged period, will result in a blunted stress response (32, 33).

The majority of the above described research has investigated HPA-axis functioning by measuring circulating cortisol in saliva, which offers insight into acute cortisol levels across the day and in response to specific stressors. In recent years, measures have been complemented by assessing cortisol concentrations in hair samples, which represent a more chronic measure of cortisol (34, 35). Cortisol circulating in the bloodstream is incorporated by the hair through passive diffusion.

Several studies have found associations between experienced maltreatment and hair cortisol levels later in life (e.g., 36, 37). A meta-analysis found evidence of elevated cortisol concentrations in hair following trauma sometimes years later (including maltreatment)—albeit with small effect sizes (38). Interestingly, this study revealed two classes of effects. The first class consisted of studies that showed hypocortisolism with a moderate effect size whereas the second class consisted of studies that showed hypercortisolism with a small effect size. While the first class of studies had larger effect sizes, it contained fewer studies: four compared to 24 in the second class. All four studies in the first class investigated child maltreatment. The second class covered a broader spectrum of traumatic experiences but also included 16 studies on child maltreatment. The entire meta-analysis included studies with populations of different ages. However, age did not moderate the effect of trauma on hair cortisol levels. Ultimately, it remained unclear which factors determine whether hair cortisol concentration is blunted or elevated following child maltreatment. In the current study we investigated the unique effects of abuse and neglect on hair cortisol levels in a sample with a wide age range.

In addition to cortisol levels, cortisone levels may be relevant in the context of maltreatment and BMI. Cortisol can be metabolized into the inactive cortisone by 11β-hydroxysteroid dehydrogenase (11B-HSD) type 2 (39). Essentially no research

has been conducted to investigate the effects of maltreatment on cortisone. Therefore, the present study also includes a measure of hair cortisone.

Stress and cortisol also play a central role in obesity. Early evidence of the causal role cortisol plays in obesity comes from studies observing weight gain in patients who are administered glucocorticoids (40–42). Additional research suggests that cortisol might be involved in fat accumulation and weight gain (43) by inducing greater food intake (44–46) and disrupting the regulation of fat storage (47). Generally, higher levels of cortisol have been linked to higher BMI (48) but there are also examples of hypocortisolism and higher BMI (49). To date, only a few studies have explicitly explored the relationship between cortisol measured in hair and obesity and most of these studies have relatively small sample sizes. Overall, these studies generally find elevated cortisol concentrations in obese children (50) and adults (51, 52) but not all studies find a relationship (53). Also, it has been suggested that the impact of cortisol on weight not only depends on how much cortisol is secreted but also how it is metabolized. The release of more cortisol may be triggered if more cortisol is transformed to cortisone (54).

The current study adds to the existing research by testing the mediating role of hair cortisol—as an objective measure of chronic stress and HPA-axis functioning—in the relation between retrospectively measured maltreatment and the BMI of children and adults. To our knowledge, only one study has investigated cortisol dysregulation as a mediator between maltreatment and BMI (55). This study found that a flatter cortisol awakening response partially mediated the effect of early adversity on BMI. Since the cortisol awakening response is not correlated to cumulative cortisol secretion measured by hair cortisol it is still unknown whether overall cortisol production measured in hair mediates the relation between maltreatment and BMI. In addition, the current study focused on the unique effects of abuse and neglect on cortisol levels and BMI in line with the theoretical proposition that they represent different types of stressors (56). It has been suggested that cortisone is an alternative, parallel measure of cortisol (35). For a better understanding of HPA axis functioning and corticosteroids in the body (57), we investigated the concentration of both the active cortisol and the inactive cortisone in hair.

We expected that abuse and neglect experienced in childhood would be associated with higher BMI at the time of the study. Further, we expected that abuse and neglect would be related to hair cortisol and cortisone concentrations and that these would in turn be related to BMI. We hypothesize that the effect of maltreatment on BMI would be mediated by elevated hair cortisol and cortisone concentrations. Given mixed results in previous research, we explored whether abuse and neglect had differential effects.

It has been argued that the initial response to experiencing maltreatment is an increased stress reaction, but that over time chronic stress results in down-regulation (30, 32, 58). In this study, we included children and adults. As a result, for some participants experiences of abuse and neglect were potentially acute if they still lived with their parents at the time of the study while it was in the past for the participants who had moved out of their parental home—in some cases decades ago. These two groups might display different HPA axis activity. Therefore, we explored whether the same associations would be observed when performing the analyses for these two groups separately. Following the allostatic load model, elevated cortisol levels would be expected in younger participants who had experienced maltreatment and blunted cortisol levels in adult participants who had experienced maltreatment. Moreover, an association between maltreatment and BMI may only arise in adults.

METHOD

Sample

The sample of the current study was drawn from the Dutch 3G Parenting Study that investigates intergenerational transmission of parenting styles, stress and emotion regulation using a multi-generational design (for details see 59, 60).

For this investigation, we included participants if they agreed to provide a hair sample and had sufficient hair growth resulting in a sample of N = 280. Of these participants, n = 31 were excluded because of corticosteroid use in the previous three months. The final sample of 249 participants came from 60 families with an average of 4.15 family members per family (range: 1 to 18), aged 8 to 87 years (M = 36.13, SD = 19.33).

Procedure

Participants attended one or two 7-hour lab visits at the Leiden University Medical Center with their nuclear families. During these lab visits, participants completed questionnaires, computer tasks, interviews, and family interaction tasks. In addition, samples of hair, saliva, and buccal cells were collected. All participants signed informed consent. Parents cosigned informed consent if the participant was under the age of 18 years. Also, maltreatment questionnaires of underage participants were inspected after each lab visit. Ethical approval was obtained from the Ethics Committee of the Leiden University Medical Centre (reference number: P11.134).

Instruments

Child Maltreatment

We measured child maltreatment experiences with a combination of two self-report questionnaires: the Parent-Child Conflict Tactics Scales (CTSPC, 61) and the Childhood Trauma Questionnaire (CTQ, 62, 63). Four maltreatment subtypes were assessed: 1) physical abuse (13 items; CTSPC), 2) emotional abuse (five items; CTSPC), 3) physical neglect (four items; CTSPC), and 4) emotional neglect (six items; CTSPC and CTQ). The CTSPC and the CTQ use different rating scales to assess frequencies. In order to be consistent across items, a 5-point scale ranging from (1) never to (5) (almost) always was used for all items. The emotional neglect items from the CTQ were reverse coded.

To measure maltreatment, we used a multi-informant approach in which we combined child- and parent-reports. All participants reported on whether they had experienced maltreatment (i.e., child-report). For the child-report score, participants reported on maternal and paternal behavior separately. Per subtype, we first calculated averages for maltreatment perpetrated by mother and maltreatment perpetrated by father. Then, per subtype, the higher score of mother or father was included in the child-report score. In addition, if a participant participated with a parent, that parent also reported on whether they had perpetrated maltreatment towards that particular participant (i.e., parent-report). The same approach was used to calculate the parent-report score. At least one parent score was available for 61% of the participants. Child-report and parent-report were averaged. Lastly, abuse and neglect scores were calculated by averaging the average for physical and the average for emotional abuse ($r(247) = .64$, $p < .001$) and the average for physical and the average for emotional neglect ($r(247) = .38$, $p < .001$), respectively. Cronbach's αs show acceptable internal consistency for child-report (abuse: $\alpha_{mother} = .92$, $\alpha_{father} = .91$; neglect: $\alpha_{mother} = .86$, $\alpha_{father} = .86$) and parent-report (abuse: $\alpha_{mother} = .78$, $\alpha_{father} = .84$; neglect: $\alpha_{mother} = .76$, $\alpha_{father} = .67$).

It should be noted that the procedure for younger participants was slightly adjusted. For participants under 12 years of age, experienced maltreatment was assessed orally and questions about very severe physical abuse were omitted. Participants who were 12 years or older and living with their parents at the time of the study indicated whether they had experienced maltreatment within the last year or in the years before. Per item, the higher score of these two was included. For the full questionnaire and further details, see **Data Sheet 1**.

Hair Cortisol and Cortisone

During the lab visit, a research assistant cut approximately 100 hairs closely from the scalp at the back of the head. In most cases, the most proximal 3 cm were analyzed. For six participants, the available hair was shorter than 3 cm: for one participant 1 cm of hair was available, for two participants 2 cm of hair, and for three participants 2.5 cm of hair. Hair samples were prepared by weighing them, cutting them in smaller pieces with surgical scissors, and washing them with 1.0 ml of LC—MS grade isopropanol for 2 min. The hair samples were incubated over night with 1.4 ml LC—MS grade methanol and in presence of 100 µl internal standard (cortisol-d4) for 18 h at 25°C while gently shaking to extract cortisol and cortisone. In line with previous research (e.g., 34, 57), we log-transformed to reduce skewness.

At a growth-rate of approximately one centimeter per month, one centimeter of hair represents the cumulative cortisol secretion of the past month (64). It is possible to reliably assess cortisol in the 3 cm of hair most proximal to the scalp, i.e., representing a retrospective measure of the last three months (35). Hair cortisol has been shown to be related to average salivary cortisol levels from repeated measures across several days but is not associated to the cortisol awakening response or the diurnal cortisol pattern (65–67). Moreover, test-retest correlations indicate that hair cortisol is more stable over time than cortisol measured in saliva or urine (65, 68).

BMI

Participants either self-reported on height and weight or, if they did not know, their height and weight were measured by a research assistant. BMI was calculated from these height and weight measures (kilograms/meters^2). BMI and age were correlated [$r(233) = .56$, $p < .001$]. When assessing BMI in children and adolescents, it has been recommended to use age and gender specific scores based on external reference values (69). Therefore, for participants younger than 21 years, raw BMI scores were transformed into standard deviation scores (SDS) based on a representative Dutch sample (70) applying the LMS method (71) in the software package *childsds* (72) implemented in R (73). For individuals of 21 years and older, no SDS were available. In order to combine scores from both age groups, adult BMI scores were transformed by regressing out age and sex, and standardizing the residuals. This combined score will be denoted as BMI-*z* in the remainder of the manuscript.

Hair Related Variables

We assessed several factors that might affect hair cortisol and cortisone levels using a questionnaire. We used open questions to ask participants about their ethnicity, medication use, and hair color. Answers were coded to match previous studies on determinants of hair cortisol and cortisone (34, 57). Ethnicity was recoded into Northern European and other, and medication use into yes or no. Hair color was recoded into black, brown, blond, red, and grey. Further, participants reported whether or not they had dyed, bleached, or permed their hair in the last 3 months, whether they washed their hair more or less than three times a week, whether they had washed their hair more or less than 24 h before taking the hair sample, and whether they had used any hair styling products on the day of sampling. Lastly, the astronomical season of the lab visit was included.

Since most of these confounders, with the exception of season, hair color, and medication, were not related to either hair cortisol or cortisone (see **Table 1**) and adding them increases the number of free parameters, we did not include them in the main analysis but we explored relevant confounders in a sensitivity analysis. All variables were dummy coded. Season was dichotomized to differentiate participants who had participated in summer compared to any other season. Information on hair dying, bleaching, and perming was summarized to indicate whether a participant had undergone any hair treatment in the past three months or not. Adding these confounders as independent variables to be regressed on hair cortisol and cortisone resulted in poor model fit ($X^2 = 142.75$ ($p = .00$, $df = 47$), RMSEA = 0.90, and CFI = 0.78). Of all the confounding variables, only season (summer vs. other season) was significantly related to cortisol ($\beta = -0.13$, $p = .03$) and medication use was related to cortisone ($\beta = .16$, $p = .02$). This is in line with the preliminary analyses (**Table 1**). Contrary to the preliminary analyses, grey hair was neither related to hair cortisol nor to cortisone—likely because the effect was explained by age.

TABLE 1 | Sample characteristics and their association with hair cortisol and cortisone.

Characteristic	Mean(SD)/ N(%)	Cortisol M (IQR)	Cortisone M (IQR)
Sex			
Male	77 (31%)	2.01 (2.36)	6.54 (5.50)
Female	172 (69%)	1.93 (2.01)	5.87 (4.80)
Age (in years)	36.13 (19.33)		
Abuse	1.57 (0.42)		
Neglect	1.83 (0.52)		
BMI	23.95 (5.40)		
Medication use			
Yes	98 (39%)	2.37 (2.93)	7.16 (6.61)
No	150 (60%)	1.82 (1.99)*	5.84 (3.77)*
Ethnicity			
Northern European	242 (97%)	1.99 (2.15)	6.10 (4.78)
Other	7 (3%)	2.68 (1.81)	7.90 (6.33)
Hair color			
Black	3 (2%)	1.65 (2.47)	5.18 (11.31)
Brown	70 (28%)	2.18 (2.21)	6.18 (5.55)
Blond (ref)	155 (62%)	1.92 (2.07)	6.06 (4.36)
Red	10 (4%)	1.54 (4.46)	5.54 (16.64)
Grey	10 (4%)	3.10 (8.95)*	10.39 (19.82)*
No hair treatment (last 3 months)	166 (67%)	1.87 (2.11)	6.20 (4.69)
Hair dyed	72 (29%)	2.27 (2.25)	5.87 (5.23)
Hair bleached	33 (13%)	2.91 (2.89)	6.78 (6.48)
Hair permed	5 (2%)	2.69 (0.86)	5.55 (2.55)
Hair washing frequency			
< 3/week	104 (42%)	1.92 (2.56)	5.86 (4.13)
≥3/week	144 (58%)	2.05 (1.84)	6.73 (5.03)
Last hair wash			
≤24 h before sampling	137 (55%)	1.88 (2.20)	6.21 (5.09)
> 24 h before sampling	111 (45%)	2.17 (2.03)	6.16 (4.39)
Use of hair styling products on day of sampling			
Yes	117 (47%)	2.21 (2.20)	6.49 (5.30)
No	130 (52%)	1.83 (1.78)	6.05 (4.38)
Season represented in hair sample			
Winter	49 (20%)	1.81 (2.40)	6.35 (3.21)
Spring	87 (35%)	1.92 (2.20)*	6.05 (5.36)
Summer (ref)	47 (19%)	2.70 (3.09)	6.26 (5.45)
Fall	66 (27%)	1.86 (1.58)*	6.03 (5.32)

Ns may vary due to missing data. For continuous variables, means and standard deviations (SD) are reported. Conversely, for categorical data, Ns and percentages are reported. For categorical data, cortisol and cortisone levels are presented as median (M) and interquartile ranges (IQR) of each group.

Therefore, a sensitivity analysis was performed only including season and medication use as potential confounders.

Demographic Information

Age and sex were included as demographic variables as well as household socioeconomic status (SES). To assess SES we asked participant of 18 years and older about their household income and highest completed education. Yearly household income was measured on a 7-point scale ranging from (1) less than € 15,000 to (7) more than € 65,000. Due to changes in the Dutch educational system, first and second generation participants rated education on a 7-point scale and third generation participants rated education on a 10-point scale. Both scales were rescaled to a 4-point scale. Based on standardized household income and standardized completed educational level a composite household SES score was calculated. If data of two partners living in the same household was available their scores were averaged for the household SES score. Children living with their parents shared their parents' household SES score.

Analysis

Outliers

Outliers (i.e., $z > \pm 3.29$) were winsorized (74) by adding or subtracting the difference between the last two acceptable raw values to the last acceptable value. We winsorized two outliers for abuse, one for neglect, four for hair cortisol, seven for hair cortisone, and one for BMI.

Missingness

The missing information were as follows: 2.4% hair cortisol, 1.2% hair cortisone, 5.6% BMI, 1.2% hair dyed, 1.2% hair bleached, 0.8% hair permed, 0.4% hair washing frequency, 0.4% on last hair wash, and 0.8% on hair product use. Little's missing completely at random (MCAR) test was not significant ($\chi 2 = 27.20$, $df = 22$, $p = 0.20$) indicating that data was MCAR. Therefore, we imputed missing values using full information maximum likelihood (FIML).

Structural Model

The following analyses were implemented in the R package lavaan 0.6-5 (75). A structural model was used to simultaneously test the effects of abuse and neglect on hair cortisol, hair cortisone, BMI-*z*, as well as the covariance between hair cortisol and BMI-*z*, and hair cortisone and BMI-*z*. Theoretically, we expect that glucocorticoids would affect BMI but methodologically we cannot infer causality in our study. By measuring cortisol and cortisone in hair we get an estimate covering the past 3 months, while BMI-*z* was measured during the test day. A model in which glucocorticoids and BMI-*z* mutually reinforce each other is plausible (76, 77). Thus, given the temporal proximity of these measures and the possibility of BMI-*z* affecting HPA-axis functioning, we tested a bidirectional relation rather than a directional effect between cortisol and cortisone on the one hand, and BMI-*z* on the other hand. SES was always included as a covariate. We controlled for age and sex in the prediction of hair cortisol and cortisone but not BMI-*z* because this score was already corrected for age and sex effects. We allowed abuse and neglect to covary with each other and cortisol and cortisone to covary with each other. Based on bivariate correlations, we also included covariation between age and abuse and neglect, as well as SES and neglect.

The model parameters are determined using a maximum likelihood estimator. Standard errors were bootstrapped (1,000 samples) because some of the variables had a non-normal distribution. To assess fit, we inspected comparative fit index (CFI) and the root mean square error of approximation (RMSEA). A CFI above .90 and an RMSEA below .06 was considered to describe a good fit between the model and the observed data (78).

TABLE 2 | Pearson correlations.

	Abuse	Neglect	Cortisol	Cortisone	BMI-*z*	Sex	Age
Neglect	.54**						
Cortisol	.16*	.12					
Cortisone	.21**	.13*	.71**				
BMI-*z*	.15*	.06	.05	.01			
Sex	.05	.01	-.04	-.12	.01		
Age	.18**	.31**	.33**	.24**	-.06	.09	
SES	-.04	-.16*	.10	.01	-.13*	.01	-.09

*Cortisol and cortisone were log-transformed; Sex: male coded as 1, and female coded as 2. *p < .05; **p < .01.*

RESULTS

Descriptive Statistics

Correlations and descriptive statistics are reported in **Tables 1** and **2**. No differences between men and women were observed but older participants had higher levels of abuse, neglect, cortisol, and cortisone (*p*s < .01). SES was negatively related to neglect and BMI-*z* (*p*s < .05). Abuse and neglect were correlated ($p < .01$) as were cortisol and cortisone ($p < .01$). Lastly, abuse was correlated to cortisol, cortisone, and BMI-*z* ($p < .05$) whereas neglect was only related to cortisone ($p < .05$). For participants who were younger than 21 years, a BMI SDS of 1 or higher was considered overweight and of 2 or higher was considered obese (79). For participants who were 21 and older, the cut-offs were raw BMI scores of 25 and 30 for overweight and obese, respectively (80). Fifty-seven participants (23%) were overweight but not obese and 35 were obese (14%). Note that we use BMI categories only for descriptive purposes here but work with continuous, standardized scores (BMI-*z*) in the analyses for optimal power (81).

Structural Model

The hypothesized model fit the observed data well with $X^2 = 7.73$ ($p = .46$, $df = 8$), RMSEA = 0.00, and CFI = 1.00. The complete model with standardized path coefficients is presented in **Figure 1**.

Pathways to BMI-z

Lower SES was associated with higher BMI-*z* scores (β = -0.13, $p = .02$). Age and sex were not included as covariates as BMI-*z* was already corrected for age and sex. There was a significant positive association between abuse and BMI-*z* (β = 0.17, $p = .04$) with higher levels of experienced abuse predicting higher BMI-*z*. The effect of experienced neglect on BMI-*z* was not significant (β = -0.05, $p = .57$). Hair cortisol and hair cortisone were not associated with BMI-*z* (cortisol: β = 0.08, $p = .22$; cortisone: β = 0.01, $p = .85$).

Pathways to Hair Cortisol

Hair cortisol concentrations were higher in older participants (β = 0.35, $p < .001$) and participants with a higher SES (β = 0.13, $p = .05$) but were not signif ica nt ly associated with sex (β = -0.07, $p = .23$). Taking into account these demographic variables, there were no significant relations with abuse (β = 0.12, $p = .06$) or neglect (β = -0.03, $p = .61$).

Pathways to Hair Cortisone

We observed higher hair cortisone levels for older participants (β = 0.24, $p < .001$) and men compared to women (β = -.13, $p = .02$). There was no association with SES (β = 0.04, $p = .16$). Controlling for age, sex, and SES, experiencing abuse was significantly related to hair cortisone. Higher levels of experienced abuse were related to greater cortisone concentrations (β = 0.19, $p < .01$). Conversely, the effect of experienced neglect was not significant (β = -0.04, $p = .56$).

Indirect Effects

An indirect effect can be significant even if not all of the direct paths are significant. For completeness, we tested whether any of the indirect effects were significant, however, this was not the case (*p*s > .38).

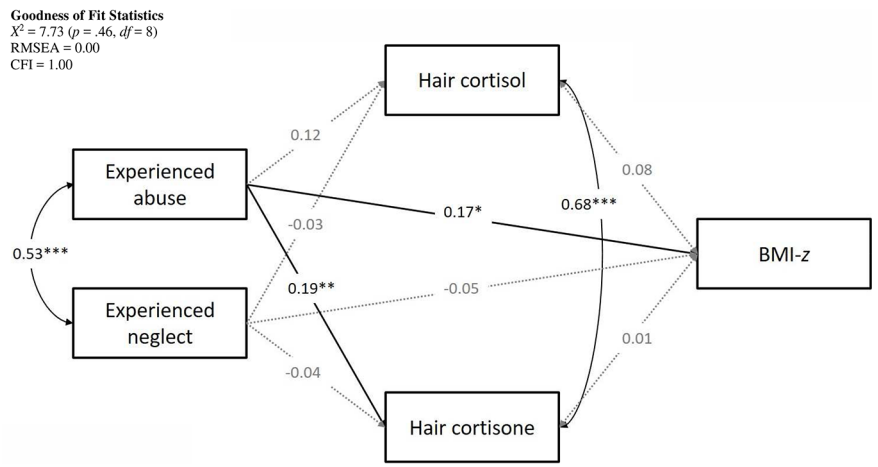

FIGURE 1 | Structural model.

Sensitivity and Exploratory Analyses

A number of additional analyses were conducted to exclude alternative explanations for the findings. For one, trauma may affect individuals in a different way depending on whether it is ongoing or not. Therefore, we ran the analyses separately for participants who were still sharing a household with their parents and those who were not. Of the total sample, 173 participants were not living with their parents at the time of the data collection. Running the model in this subsample, we found good model fit [X^2 = 7.61 (p =.47, df = 8), RMSEA = 0.00, and CFI = 1.00]. We found the same pattern of significant results and similar strength and direction of path coefficients. There were 76 participants who were living with their parents at the time of the data collection. The model fit for this subsample was acceptable [X^2 = 5.79 (p =.67, df = 8), RMSEA = 0.00, and CFI = 1.00]. However, running the path model for this subgroup led to a different pattern of results with the previously significant effects of abuse on hair cortisone concentration (β = 0.14, p =.35) and BMI-z (β = 0.02, p =.92) disappearing. This might suggest an age effect but could also be the result of a lack of power in the last analysis as this group was smaller than the group of participants who were not living with their parents.

We initially tested the unique effects of cortisol and cortisone. Cortisol and cortisone were highly correlated. In two sensitivity analyses we tested whether cortisone suppressed the effects of cortisol and vice versa by running the analyses for cortisol and cortisone separately. In both cases the estimates of model fit were satisfactory [cortisol only: X^2 = 7.71 (p =.46, df = 8), RMSEA = 0.00, and CFI = 1.00; cortisone only: X^2 = 7.62 (p =.47, df = 8), RMSEA = 0.00, and CFI = 1.00] and the same pattern of significant results emerged, with similar path coefficients, suggesting that there was no suppression effect. It has also been suggested that the cortisol:cortisone ratio and the sum of cortisol and cortisone may be meaningful indicators of HPA-axis activity (57). Therefore, we ran two additional models for the ratio and the sum. Both models had good model fit [ratio: X^2 = 7.71 (p =.46, df = 8), RMSEA = 0.00, and CFI = 1.00; sum: X^2 = 7.64 (p =.47, df = 8), RMSEA = 0.00, and CFI = 1.00]. Ratio was predicted by age (β =.24, p =.001) and SES (β =.14, p =.03) but not abuse or neglect. Ratio and BMI-z were also not related (β =.10, p =.10). The sum of cortisol and cortisone was related to age (β =.32, p < .001) and abuse (β =.16, p =.02) but not BMI-z (β =.03, p =.64).

Physical and emotional maltreatment might have differential effects. Therefore, we explored physical and emotional abuse, and emotional neglect in separate models. We omitted physical neglect from these analyses because on its own, physical neglect did not have sufficient variance (M = 1.19, SD = 0.28) or internal reliability (α =.24–.64). For physical abuse, model fit was good [X^2 = 2.16 (p =.34, df = 2), RMSEA = 0.02, and CFI = 0.99]. Physical abuse showed similar associations with the variables in the model as overall abuse did. There was a significant association with BMI-z (β =.15, p =.02), cortisol (β =.12, p =.05) and cortisone (β =.16, p =.01). Results were also similar for emotional abuse [model fit: X^2 = 2.15 (p =.34, df = 2), RMSEA = 0.02, and CFI = 0.99]. Emotional abuse was associated with BMI-z (β =.16, p =.02), cortisol (β =.11, p =.05), and cortisone (β =.16, p =.01). The pattern of results for emotional neglect was also similar to overall neglect. Model fit was adequate [X^2 = 1.91 (p =.39, df = 2), RMSEA = 0.00, and CFI = 1.00]. Emotional neglect was not associated with any variables of interest (BMI-z: β =.02, p =.74; cortisol: β =.03, p =.58, cortisone: β =.07, p =.07).

We also ran the model again with season and medication use as control variables for hair cortisol and cortisone, respectively. This resulted in a model that fit the data adequately [X^2 = 37.86 (p =.01, df = 20), RMSEA = 0.60, and CFI = 0.95] and did not affect the path coefficients substantially.

DISCUSSION

This study investigated the effect of experiencing child maltreatment on BMI and the mediating role of chronic stress, indexed by hair cortisol and cortisone. Confirming previous research (12, 13), we found a relation between reported experiences of abuse and higher BMI-z. Moreover, we observed higher concentrations of hair cortisone in individuals who had experienced abuse. However, contrary to our expectations, neither hair cortisol nor hair cortisone mediated the association between abuse and BMI-z. Neither hair cortisol nor hair cortisone had a direct effect on BMI. Moreover, neglect did not affect hair cortisol, hair cortisone, or BMI-z.

In line with most previous research (12, 13), abuse was related to higher BMI. This finding indicates that early experiences can initiate a trajectory that results in weight gain later on and implicates psycho-emotional risk factors in the etiology of overweight. Accordingly, recent models of obesity put greater emphasis on affective aspects of weight gain (76).

As expected, abuse was related to increases in hair cortisone (and trend-level increases in cortisol) suggesting chronic levels of stress in individuals with abuse experiences – in some cases decades after the abuse occurred. This is also in line with the majority of research on maltreatment and hair cortisol which suggest elevated cortisol secretion (38). It is interesting to contrast these findings with evidence of hypocortisolism in maltreated individuals. For instance, meta-analytic evidence suggests that in response to a social stressor, cortisol levels are blunted rather than increased in maltreated individuals. Since hair cortisol represents a cumulative measure of cortisol secretion, this might suggest that abused individuals encounter or perceive more stressful events than non-maltreated individuals—even if their stress response to the particular event is blunted. Alternatively, basal cortisol and cortisone levels may be higher in individuals who experienced childhood abuse.

Evidence of hypocortisolism has generally been interpreted in the context of the allostatic load model: over time the stress response is downregulated to maintain homeostasis. The current study found evidence of elevated cortisone levels in abused individuals—in particular in adults. However, this is not necessarily in contradiction to the allostatic load model since intra-individual cortisone levels may have been downregulated

over time, but the cross-sectional nature of the study does not allow us to test this hypothesis. Collectively, these findings show that experiences of abuse get embedded in the body's physiology by reprogramming HPA-axis and higher BMI but that these might be separate processes.

From a methodologic perspective, this finding also supports the measurement of hair cortisone in addition to hair cortisol. At the moment, the majority of studies focus on hair cortisol and do not include a hair cortisone measures. Cortisone levels are generally higher than cortisol levels. It has been suggested that this might make cortisone more readily detectable and a more powerful indicator of chronic stress (57). Effects of abuse on cortisol seem to parallel the effects on cortisone but might require a larger sample size than cortisone. Given that it can be difficult to achieve large sample sizes when studying specific populations, measuring hair cortisone could hold a lot of promise. Moving forward, it will be important to gain a better understanding of the relation between cortisol and cortisone in general and in hair specifically. The correlation between cortisol and cortisone is indicative of a strong relation but also suggests that they are not entirely interchangeable. Functionally, cortisone is less active than cortisol but it is nevertheless an important indicator of HPA axis activity since cortisone and cortisol are closely linked through the 11B-HSD enzymes. Cortisol can be metabolized into cortisone by 11B-HSD type 2 and cortisone can be transformed back into cortisol by 11B-HSD type 1. Therefore, it has been suggested that including measures of 11B-HSD type 1 and 2 activity could provide a completer picture of HPA axis functioning (82). On an endocrine level, 11B-HSD may be involved in the HPA axis feedback loop and contribute to the daily production of cortisol (83).

Our results suggest that the association between childhood abuse and later BMI is not mediated by hair cortisol and cortisone. This can be attributed to the fact that, contrary to our expectations, hair cortisol and cortisone were not associated with BMI-*z*. Previous research has suggested a relation between HPA-axis dysregulation and obesity but results were inconsistent (84). More sensitive measures to assess the relation between HPA-axis functioning and obesity might be site-specific cortisol activity such as intra-adipocyte cortisol concentrations and abdominal adiposity measures to assess body fat distribution (48, 85). Also, differences in hair cortisol may only arise at higher levels of BMI. For instance, one study found that obese participants had higher hair cortisol levels compared to overweight and normal weight participants but no difference between overweight and normal weight participants (52). In the current study, 14% of the sample was obese whereas the majority (63%) was normal weight.

Stress may affect men and women differently. While there is meta-analytic evidence suggesting that the effect of maltreatment on obesity is stronger in women, another meta-analysis suggested that psychosocial stress encountered in adulthood has a stronger effect on BMI in men (86). The same study found stronger effects of psychosocial stress on obesity if the follow-up period was longer. In the current study, two-thirds of the participants were women. Hair cortisol and cortisone measures cover a period of three months whereas years or decades may have elapsed since experiencing abuse. Taken together, these details might explain why we observed an association between abuse and BMI-*z* but no effect of cortisol and cortisone on BMI-*z* because abuse would affect women more strongly and has a longer follow-up period.

Addressing the affective component of obesity could have profound consequences because traditional weight loss interventions primarily focus on non-affective causes by modifying the energy balance by decreasing energy intake and increasing energy expenditure. And while interventions focusing on diet and exercise have been shown to reliably reduce weight, their effects on weight loss are small (87–89). Making matters worse, participants struggle to maintain weight loss and often drop out without completing the intervention (90, 91). For individuals with childhood abuse experiences, these traditional approaches to weight loss may not be sufficient because they do not fully address the underlying problem.

An important issue to consider concerns the timing of interventions. It appears to be generally true that early prevention is more effective than later intervention because smaller changes in behavior are necessary to maintain than to lose weight (92). In the present study, there was no association between abuse and BMI in the younger subgroup who were still living with their parents and are therefore still at risk of parent-to-child abuse. This result should be interpreted with some caution since this subgroup was small. It is interesting to note that in the younger subgroup the association between abuse and cortisone remained similar in strength even though it was not significant anymore. However, the association between abuse and BMI essentially dropped to zero—possibly a sign that this is not solely a power issue. This is in line with meta-analytic evidence showing no effect of maltreatment on BMI in children and adolescents (12). Taken together, this indicates that childhood abuse might be an early risk factor for obesity but the effects may only become apparent later on. Early identification of groups who are at heightened risk for developing overweight is a crucial component of effective prevention.

It may also be relevant to explore other mediating factors such as eating as coping behavior. Research shows that abused individuals are more likely to a develop binge eating disorder (93). This may be an attempt to regulate negative emotions through emotional eating (10, 94). In line with this assumption, symptoms of depression and post-traumatic stress have been found to mediate the relation between maltreatment and obesity —but the evidence is tenuous (17, 27). It has also been argued that unhealthy eating patterns as a result of maltreatment may be associated with decreases in prefrontal cortex volume and less inhibitory control (12). One study has found that sexual abuse resulted in higher BMI in impulsive individuals only. However, the moderating effect of impulsivity was not found for other types of maltreatment (95). More research is needed to explore the mechanisms underlying the effect of maltreatment on BMI.

In the current study, neglect was not related to BMI. The type of maltreatment might affect how the HPA-axis is

reprogrammed. It has been argued that abuse and neglect can be differentiated along two dimensions of adverse childhood experiences: abuse representing threat and neglect representing deprivation (96, 97). Threat may not elicit the same stress response as deprivation. For instance, one study found that adolescents who had experienced abuse had chronically elevated stress levels, whereas adolescents who had experienced neglect had blunted stress levels (cited in 98), possibly because threat requires activation of the stress system whereas deprivation does not. Another explanation for this finding might be that participants reported little physical neglect. Therefore, the neglect score primarily represents emotional neglect. Previous evidence suggests that emotional neglect might not be related to higher BMI and obesity (12). It would be interesting to explore how emotional neglect, specifically, differs from the other types of maltreatment. Importantly, these results do not suggest that emotional neglect is not a relevant adverse childhood experience. Rather, these findings suggest that it is time to move away from a purely cumulative model which ignores the type of experience (56). Different types of maltreatment and adverse childhood experiences might affect different developmental pathways in different ways. For instance, emotional neglect might not disrupt stress pathways such as HPA-axis development but it may be detrimental to learning pathways such as reward learning because it deprives the child of positive and sensitive emotional input (56, 99).

Limitations

The current study had several shortcomings. We did not assess during which developmental period maltreatment occurred. It has been suggested that neglect during the first two to three years of life is particularly crucial in programming HPA-axis functioning (37, 98). Our assessment of maltreatment does not allow us to discern between neglect starting early in the development and neglect that occurred later in the development. Moreover, at least in child reports, any maltreatment that occurred in the first three to four years is unlikely to be remembered and hence, unlikely to be reported (100). Another consequence of not knowing the exact timing of maltreatment experiences is that we could not include the exact time that had elapsed since maltreatment as a potential moderator.

Moreover, the cross-sectional nature of this study means that we cannot draw any conclusion on the causality of the associations. Child maltreatment was assessed retrospectively and thus current stress levels may have affected participants' responses. Generally, agreement between prospective and retrospective measures of child maltreatment tends to be low (101). Baldwin and colleagues (101) argue that prospective and retrospective measures are not interchangeable, but both are valuable. The advantage of retrospective reports is that they may capture more true cases as prospective reports often rely on official records which often underestimate incidence of maltreatment and only capture the most extreme cases. Moreover, cortisone levels are unlikely to account for the association between abuse and cortisone entirely —especially since we used multi-informant scores of maltreatment for most of the participants. What is more, if maltreatment scores were driven by current cortisone levels, we would expect this to affect neglect scores as much as abuse scores which was not the case. Hair cortisol and cortisone levels represent the cumulative secretion of glucocorticoids over the three months prior to measuring BMI. This close temporal proximity makes it difficult to draw strong conclusions with regards to the causal effect of cortisol and cortisone on BMI. For this reason, we tested a bidirectional effect. Since this association was not significant we can conclude that in the present study there was no relation between cortisol and cortisone on the one hand, and BMI on the other hand.

Lastly, BMI is the most commonly used metabolic outcome and is intended to, indirectly, assess body fat percentage (102). However, it is a crude measure and there might be better ways to assess overweight. One disadvantage of BMI is that it is confounded by physical fitness (103, 104). Due to the ease of assessing BMI compared to more direct measures of body fat percentage, it is unlikely to disappear soon. It also allows the comparison with population norms and other studies. For future research, it might be interesting to incorporate other indicators of metabolic health such as leptin deficiency (105) in addition to BMI. It should also be noted, that in some cases, BMI was based on self-report which may introduce some imprecision.

Conclusion and Implications

Experiencing physical and emotional abuse during childhood can have negative consequences which in some cases are still present decades later. The current study found that experienced abuse was related to elevated levels of hair cortisone and higher BMI. Both elevated cortisone, as a measure of chronic stress, and higher BMI may be related to further negative outcomes and risks such as hypertension, cardiovascular disease, and inflammation (106–110). These findings emphasize that interventions for weight-loss can benefit from integrating psychological components that address stress specifically—especially in individuals with trauma history. Alternatively, finding ways to increase (low to moderate intensity) physical activity might be an accessible way to address both imbalance in the HPA-axis (111) and reduce health risks associated with overweight and obesity (112). The current study found no effect of neglect on cortisol or cortisone or BMI. More and more research is showing differential effects of abuse and neglect (113). This suggests that treatment should be tailored to the specific type of maltreatment individuals have experienced, and we that should not expect that one size fits all.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Leiden University Medical Centre. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

The 3G Parenting Study was conceptualized by MI, BE, and MB-K. KP, LB, RB, and LC-B performed the data collection. KP and LA developed the research question. KP performed the statistical analyses and wrote the manuscript with input from all the 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.2020.00387/full#supplementary-material.

DATA SHEET 1 | Child Maltreatment Instrument.

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Analysis of GWAS-Derived Schizophrenia Genes for Links to Ischemia-Hypoxia Response of the Brain

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Obstetric complications (OCs) can induce major adverse conditions for early brain development and predispose to mental disorders, including schizophrenia (SCZ). We previously hypothesized that SCZ candidate genes respond to ischemia-hypoxia as part of OCs which impacts neurodevelopment. We here tested for an overlap between SCZ genes from genome-wide association study (GWAS) (n=458 genes from 145 loci of the most recent GWAS dataset in SCZ) and gene sets for ischemia-hypoxia response. Subsets of SCZ genes were related to (a) mutation-intolerant genes (LoF database), (b) role in monogenic disorders of the nervous system (OMIM, manual annotations), and (c) synaptic function (SynGO). Ischemia-hypoxia response genes of the brain (IHR genes, n=1,629), a gene set from RNAseq in focal brain ischemia (BH, n=2,449) and genes from HypoxiaDB (HDB, n=2,289) were overlapped with the subset of SCZ genes and tested for enrichment with Chi-square tests ($p < 0.017$). The SCZ GWAS dataset was enriched for LoF (n=112; p=0.0001), and the LoF subset was enriched for IHR genes (n=25; p=0.0002), BH genes (n=35; p=0.0001), and HDB genes (n=23; p=0.0005). N=96 genes of the SCZ GWAS dataset (21%) could be linked to a monogenic disorder of the nervous system whereby IHR genes (n=19, p=0.008) and BH genes (n=23; p=0.002) were found enriched. N=46 synaptic genes were found in the SCZ GWAS gene set (p=0.0095) whereby enrichments for IHR genes (n=20; p=0.0001) and BH genes (n=13; p=0.0064) were found. In parallel, detailed annotations of SCZ genes for a role of the hypoxia-inducible factors (HIFs) identified n=33 genes of high interest. Genes from SCZ GWAS were enriched for mutation-intolerant genes which in turn were strongly enriched for three sets of genes for the ischemia-hypoxia response that may be invoked by OCs. A subset of one fifth of SCZ genes has established roles in monogenic disorders of the nervous system which was enriched for two gene sets related to ischemia-hypoxia. SCZ

genes related to synaptic functions were also related to ischemia-hypoxia. Variants of SCZ genes interacting with ischemia-hypoxia provide a specific starting point for functional and genomic studies related to OCs.

Keywords: schizophrenia, gene-environment (G-E) interaction, obstetric complications, ischemia, hypoxia, HIF, gene expression, synapse

INTRODUCTION

A core concept of contemporary psychiatric research is that multiple genes interact with environmental factors to increase the risk of psychosis spectrum disorder, including schizophrenia (1–4). Obstetric complications (OCs) are established risk factors for schizophrenia (5–7) while it is generally thought that perturbations of oxygen and substrate delivery (i.e., ischemia-hypoxia) during OCs affect the developing brain and impair neuronal development during critical phases (8, 9). Immune mechanisms and neuroinflammation are also considered as major prenatal risk factors for schizophrenia (10), and inflammation typically invokes hypoxic challenges (11). Furthermore, evidence for links between vascular factors, including angiogenesis, and schizophrenia has been reported (12–14).

We previously hypothesized that candidate genes for schizophrenia interact with ischemia-hypoxia during OCs (9). To explore this hypothesis, we used our "ischemia-hypoxia response" (IHR) database that combined information for changes at the mRNA level after brain ischemia-hypoxia from multiple experimental studies (15, 16). Candidate genes for schizophrenia were then annotated using the IHR gene database (9, 17). Thereby, the assumptions were made that those genes observed experimentally in the adult brain are likely expressed in the developing brain, and that genes transcribed in mice or rats are also expressed in the human brain. The concept was extended to the expression or function in vascular cells, because ischemia-hypoxia will immediately invoke vascular responses and genetic mechanisms affecting vessels may cause ischemia-hypoxia. The hypothesis was that genetic variants of schizophrenia genes annotated as IHR genes and/or vascular genes may alter responsiveness to ischemia-hypoxia during OCs. In fact, our hypothesis has received support from studies where the interaction between SNPs for selected candidate genes were linked to IHR genes and OCs in relation to schizophrenia risk (18–20).

The initial analysis (9) was based on a selection from SzGene which collected more than 700 candidate genes for schizophrenia (21). A bias within SzGene toward genes of major interest for brain damage and neurodegeneration would have inflated the importance of IHR genes. Genome-wide studies such as GWAS are now considered as standard for the hypothesis-free analysis of polygenic disorders such as schizophrenia. Therefore, it was timely to examine the genes in loci defined by GWAS in schizophrenia (22, 23) in relation to ischemia-hypoxia. To do this, we linked the schizophrenia GWAS dataset to ischemia-hypoxia using the following approaches to form relevant subsets. 1) A large set of mutation-intolerant genes (LoF) was recently described (24), and it was reported that common schizophrenia alleles in GWAS are enriched for mutation-intolerant genes (23). It was hypothesized that IHR genes should be also enriched in datasets of LoF if they are essential for the response to ischemic-hypoxic challenges. Then, tests were performed for an overlap between schizophrenia genes from GWAS, mutation-intolerant schizophrenia genes, and IHR genes. 2) Schizophrenia genes from GWAS were annotated for known monogenic disorders of the nervous system that affect development and function, and then tested for overlap with IHR genes. 3) Since schizophrenia genes emerging from genomic studies are closely related to synaptic functions (25, 26), a novel database of synaptic proteins [SynGO; (27)] was used to define IHR genes related to synaptic functions and tested for the overlap with schizophrenia genes. 4) Studies were supplemented by analyzing a recently proposed set of 104 schizophrenia candidate genes from the original 108-loci study using a complex combination of parameters (28). 5) To test for a more general involvement of neurodevelopmental disease mechanisms, a gene set related to developmental delay and autism spectrum disorder (29) was tested for enrichment as well. To further explore a to link to ischemia-hypoxia, a gene set from a recent RNAseq study in focal brain ischemia (30) and the HDB database (31) were employed.

MATERIALS AND METHODS

An overview of the analysis is provided in **Figure 1**.

Databases for IHR

The database of "ischemia-hypoxia response" (IHR) genes contains manually curated genes (n=1,629) from n=24 microarray studies in experimental brain ischemia-hypoxia. This cumulative database was first described in a study of retinal genes (15) and an update has been reported (16). Most of the genes are upregulated (higher mRNA levels) in experimental studies. The definition of "response" relates to regulation of gene expression but also includes other mechanisms, i.e. mRNA splicing, stability, and degradation. The curation included alignment of mouse or rat gene symbols with human gene symbols; this was initially achieved by fusing the IHR gene list with a human gene list (32) and then by resolving conflicts by detailed searches in Entrez Gene and HomoloGene. BLAST searches for the microarray probe were used in some cases. We estimate that the combined IHR genes (n=1,629) represent 9% of genes potentially expressed during

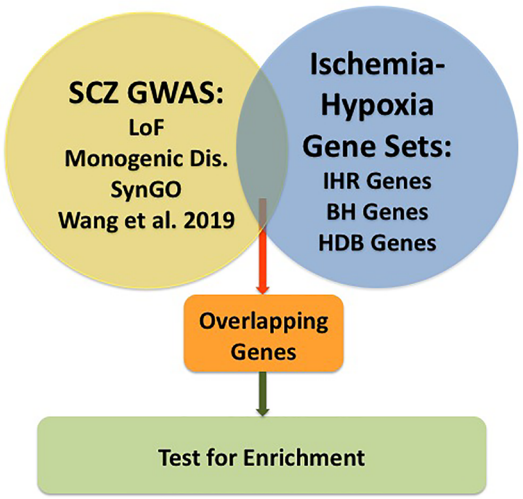

FIGURE 1 | Graphic presentation of the annotation process. GWAS data for schizophrenia (SCZ GWAS) were taken from Pardinas et al. [ref. (23)] and subsets of genes were formed for Loss of Function [LoF; ref. (24)], for Monogenic Disorders (Monogenic Dis.; described under Materials and Methods), for synaptic genes collected in SynGO [ref. (27)] and for a subset described by Wang et al., 2019 [ref.(28)]. Gene sets related to Ischemia-Hypoxia were taken from our database named "ischemia-hypoxia response genes" [IHR Genes; refs. (16, 17)], from the RNAseq study of Bhattarai et al., 2019 [BH Genes; ref. (30)] and from the public database named HypoxiaDB [HDB Genes; ref. (31)]. Overlapping genes were determined, and enrichment tested using Chi-square tests.

brain development; this was based on a conservative estimate of n=20k for the total number of protein-coding genes in the genome, the report for >90% of genes being expressed in the developing human brain (33), and on the assumption of genome-wide coverage of the microarrays. Then enrichment in a specific schizophrenia gene set was estimated by comparing the observed overlap with the randomly expected overlap set at 9%.

A recent study of focal brain ischemia in mice has used RNAseq analysis at three different time points (30); these data were combined as "BH-genes," n=2,449 (14%). Since this dataset uses the sensitive RNAseq method and a single experimental model, it was considered as a replication test for the microarray-based, cumulative dataset of IHR genes.

HDB is a database related to proteomic findings for hypoxia in general with n=2,289 entries [12%; ref. (31)]. This database is composed of findings made at the protein level across multiple organs (31) and therefore is independent of expression in the brain. For convenience, this dataset was named "HDB genes" in this analysis.

Selection of Subsets of GWAS-Based Schizophrenia Genes

N=458 genes from 145 loci of the recent GWAS dataset in schizophrenia were retrieved (23). Subsets of genes were then selected using specific annotations.

The database of loss of function (LoF) (24) was used to retrieve mutation-intolerant genes (n=3,230).

A manual annotation of GWAS-based schizophrenia genes (n=458 genes from 145 loci) was carried out for monogenic disorders that are known to affect development and function of the nervous system. Gene symbols were searched in OMIM™ (https://www.omim.org), the DDD-database (34), or DisGeNET (35); this was supplemented by abstract searches in PubMed. Genes were included when a single gene mutation has been described to cause a neurodevelopmental disorder, intellectual disability, neurological disease, or diseases of the retina and hearing nerve.

SynGO (27) was used to define synaptic proteins (n=1,112) among GWAS-based schizophrenia genes.

A subset of schizophrenia genes from GWAS analysis was defined in a recent genomic study that used a Bayesian framework to integrate multiomics data and gene networks [n=104 genes; ref. (28)].

Neurodevelopmental Disorders

To test whether the overlap with ischemia-hypoxia can be extended to neurodevelopmental disorders, a collection of genes defined by *de novo* mutations in developmental delay and autism spectrum disorder (n=253) was adopted, named DD/ASD (29).

Exploration of Hypoxia-Inducible Factors

Hypoxia-inducible factors (HIFs) are major transcriptional regulators of the hypoxia response (36, 37), including the brain (38). For annotation of schizophrenia genes, information for HIF-targets and HIF-regulators was compiled from multiple databases and literature.

Analyses

Subsets of schizophrenia genes (as defined above) and DD/ASD were overlapped with the three gene sets for response to ischemia-hypoxia. Testing for enrichment was performed by comparing the observed number of shared genes to the randomly expected number of genes using Chi-square tests ($p < 0.017$ was considered as statistically significant, given the three comparisons). As additional test for a link to synaptic genes, the IHR gene dataset was subjected to annotation by DAVID Bioinformatics (https://david.ncifcrf.gov).

RESULTS

The overall goal of this study was to uncover links between experimentally defined genes of the IHR (as collected in three databases, i.e., IHR, BH, and HDB) and genes from schizophrenia GWAS. Following the analysis of the total gene set, four subsets of genes from schizophrenia GWAS were formed and tested for enrichment. For comparison, a gene set for neurodevelopmental disorders was subjected to the same analysis.

Total Gene Set From Schizophrenia GWAS

Some genes well-known from experimental studies of brain ischemia-hypoxia stood out following the annotation of the total gene set from schizophrenia GWAS for IHR genes (**Table 1**). N=51 IHR genes, n= 59 BH genes, and n=48 HDB genes were overlapping with schizophrenia genes; however, none of these individual datasets showed enrichment in the total schizophrenia GWAS dataset. Therefore, subsets of the GWAS datasets were formed using criteria specified above and tested for enrichment with gene sets related to ischemia-hypoxia.

Subset for LoF

LoF genes were analyzed with the rationale that these are high-risk genes for schizophrenia (23). When the overall LoF database was compared with the IHR gene database, enrichment was found (n=452 or 28%; Chi-square p=0.0001), indicating that a large portion of IHR genes are potentially mutation-intolerant. The schizophrenia GWAS dataset as such was enriched with N=112 LoF genes (p=0.0001), as to be expected (23); thereby, n=25 IHR genes were among the LoF genes for schizophrenia, indicating enrichment (p=0.0002). When BH genes (30) were tested, n=35 genes were matched to LoF genes in schizophrenia (p=0.0001). Further, n=23 HDB genes were overlapping LoF genes in schizophrenia (p=0.0005). Taken together, a subset of schizophrenia genes can be defined by the combination of LoF and potential for change in expression levels during ischemic-hypoxic challenges.

Subset for Monogenic Disorders

In a separate approach, GWAS-derived schizophrenia genes were selected if a mutation was known to affect development or function of the nervous system. This annotation lead to n=96 genes (21%) of the schizophrenia GWAS dataset linked to a monogenic disorder of the nervous system. Therein, genes from the IHR dataset (n=19; p=0.008) and BH dataset (n=23; p=0.002) were enriched in the subset of schizophrenia genes matching a monogenic disorder, whereas the HDB dataset showed only a trend (n=16; p=0.05).

Subset for Synaptic Proteins

In view of multiple findings related to neurotransmission and synaptic functions in the literature for genomics of schizophrenia (25), synaptic genes within the schizophrenia GWAS dataset were analyzed in the next step. To do this, a gene/protein set curated for synaptic functions (n=1,112) was downloaded from SynGO (27). First, we tested whether synaptic genes are enriched among the IHR genes and found n=270 (24%; p=0.0001) shared genes which indicates that synaptic genes are strongly involved in the response to ischemia-hypoxia as such. For confirmation, a DAVID Bioinformatics analysis of the complete IHR gene set was run; the Gene Ontology (GO) term "synapse" (GO:0045202) was found highly enriched (p=2.2E-25, Bonferroni corrected p=1.22E-22). Next, we tested whether synaptic genes are enriched in the schizophrenia GWAS gene set as such and found n=46 shared genes (p=0.0095). Finally, when

TABLE 1 | Selected schizophrenia genes derived from genome-wide association study (GWAS) matching well-recognized ischemia-hypoxia response (IHR) genes of the brain.

Gene symbol	Official full name	Main biological function
AKT3	AKT serine/threonine kinase 3	Serine/threonine protein kinase, growth factor signaling
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2	Intracellular calcium pump associated with ER
BNIP3L	BCL2 interacting protein 3 like	Pro-apoptotic factor within the Bcl-2 family
CACNA1C	Calcium voltage-gated channel subunit alpha1 C	Alpha-1 subunit of a voltage-dependent calcium channel
CACNB2	Calcium voltage-gated channel auxiliary subunit beta 2	Subunit of a voltage-dependent calcium channel protein
CLU	Clusterin	Secreted chaperone
FGFR1	Fibroblast growth factor receptor 1	Growth factor signaling
FURIN	Furin, paired basic amino acid cleaving enzyme	Subtilisin-like proprotein convertase
HSPA9	Heat shock protein family A (Hsp70) member 9	Heat shock protein 70 gene family, primarily mitochondrial
HSPD1	Heat shock protein family D (Hsp60) member 1	Member of the chaperonin family, mitochondrial, HSP60
HSPE1	Heat shock protein family E (Hsp10) member 1	Major heat shock protein, HSP10
INA	Internexin neuronal intermediate filament protein alpha	Intermediate filament protein, in axonal cytoskeleton
MDK	Midkine	Secreted growth factor
MEF2C	Myocyte enhancer factor 2C	Transcription enhancer, trans-activating, DNA binding activities
NCAN	Neurocan	Modulation of cell adhesion and migration
NGEF	Neuronal guanine nucleotide exchange factor	Guanyl-nucleotide exchange factor activity
NRGN	Neurogranin	Postsynaptic protein kinase, binding calmodulin
OPCML	Opioid binding protein/cell adhesion molecule like	Cell adhesion, accessory role in opioid receptor function
PTK2B	Protein tyrosine kinase 2 beta	Protein tyrosine kinase, regulation of ion channels
RANGAP1	Ran GTPase activating protein 1	Regulation of nuclear transport, GTP-binding and exchange
RELA	RELA proto-oncogene, NF-kB subunit	NF-kappa-B transcription factor complex
SERPING1	Serpin family G member 1	Regulation of the complement cascade
SF3B1	Splicing factor 3b subunit 1	Splicing factor, component of U2 snRNP
SREBF1	Sterol regulatory element binding transcription factor 1	Transcription factor for sterol regulatory element-1 (SRE1)
SRPK2	SRSF protein kinase 2	Splicing factor, protein serine/threonine kinase activity

overlapping these n=46 schizophrenia genes listed in SynGO with IHR genes, n=20 were matched (p=0.0001); n=13 for BH genes (p=0.0064); and n=6 for HDB genes (p=0.6).

Subset of Multiomics Data

Subsequently, additional gene sets derived from schizophrenia GWAS data were searched for confirmation, and the dataset of n=104 genes related to schizophrenia by multiomics studies (28) was selected. Enrichment was found for IHR genes (n=31; p=0.0001), but not for BH genes (n=27; p=0.03) or HDB genes (n=22; p=0.095).

Neurodevelopmental Disorders

Furthermore, it was explored whether the role of IHR genes can be extended more broadly to neurodevelopment disorders, and a gene set for DD/ASD was analyzed (n=253) (29). Thereby, only 14/235 of the DD/ASD genes were overlapping with the schizophrenia GWAS dataset, indicating an independent dataset. Strong overlap was observed for the DD/ASD gene set with IHR genes (n=47; p=0.002), the BH dataset (n=72; p=0.0001), and HDB genes (n=59; p=0.0007).

Exploration of HIFs

HIFs are major regulators of gene expression in response to hypoxia (36, 37). Therefore, an initial survey of GWAS-based schizophrenia genes for links to HIF regulation was performed using expert curation (38). Specific annotations for HIF-related genes in the schizophrenia GWAS dataset yielded n=33 genes (**Table 2**).

DISCUSSION

Gene-environment interactions (G × E) play a key role in the neurodevelopmental model of schizophrenia. OCs are established environmental factors linked to the risk of schizophrenia which may involve ischemic-hypoxic events in the developing brain (5–7). The goal of this study was to apply datasets of experimentally defined genes responding to ischemia-hypoxia (IHR genes) to a large set of GWAS-defined schizophrenia genes. Thereby, the annotation for IHR genes opens the possibility that a matched schizophrenia gene is subject to regulation by ischemic-hypoxic periods occuring

TABLE 2 | Annotation of schizophrenia genes derived from genome-wide association study (GWAS) for a role of hypoxia-inducible factors (HIFs).

Gene symbol	Official full name	Main biological function
ALDOA	Aldolase, fructose-bisphosphate A	Glycolytic enzyme
ALPK3	Alpha kinase 3	Protein serine/threonine kinase
BNIP3L	BCL2 interacting protein 3 like	Pro-apoptotic factor within the Bcl-2 family
BTG1	BTG anti-proliferation factor 1	Regulator of cell growth and differentiation
CDK2AP1	Cyclin dependent kinase 2 associated protein 1	Role in cell-cycle and epigenetic regulation
CPEB1	Cytoplasmic polyadenylation element binding protein 1	Regulation of mRNA translation
CPT1C	Carnitine palmitoyltransferase 1C	Regulation of beta-oxidation
CREB3L1	cAMP responsive element binding protein 3 like 1	Transfactor activated by ER stress
CUL3	Cullin 3	Role in polyubiquitination
EP300	E1A binding protein p300	Histone acetyltransferase, regulation of transcription
ESRP2	Epithelial splicing regulatory protein 2	Splicing regulator
FGFR1	Fibroblast growth factor receptor 1	Growth factor signaling
FURIN	Furin, paired basic amino acid cleaving enzyme	Subtilisin-like proprotein convertase
GPR135	G protein-coupled receptor 135	Orphan receptor
HSPA9	Heat shock protein family A (Hsp70) member 9	Heat shock protein 70 gene family, mitochondrial
KAT5	Lysine acetyltransferase 5	Histone acetyl transferases, DNA repair
KDM4A	Lysine demethylase 4A	Trimethylation-specific demethylase, repressor
KMT5A	Lysine methyltransferase 5A	Protein-lysine N-methyltransferase, SETD8
LRP1	LDL receptor related protein 1	Low-density lipoprotein receptor
LSM1	LSM1 homolog, mRNA degradation associated	Pre-mRNA splicing, mediating U4/U6 snRNP formation
MAD1L1	Mitotic arrest deficient 1 like 1	Role in mitotic spindle-assembly checkpoint, cell cycle
NEK1	NIMA related kinase 1	Serine/threonine kinase, cell cycle
NMB	Neuromedin B	Bombesin-like family of neuropeptides
OGFOD2	2-oxoglutarate and iron dependent oxygenase domain containing 2	Oxidation-reduction process
OTUD7B	OTU deubiquitinase 7B	Deubiquitinase
PGM3	Phosphoglucomutase 3	Glycogen formation and utilization
PPP2R2A	Protein phosphatase 2 regulatory subunit Balpha	Negative control of cell growth and division
PRMT1	Protein arginine methyltransferase 1	Protein arginine N-methyltransferase
RALGAPA2	Ral GTPase activating protein catalytic alpha subunit 2	GTPase activator
RPTOR	Regulatory associated protein of MTOR complex 1	Interaction with mTOR kinase, negative regulator
SF3B1	Splicing factor 3b subunit 1	Splicing factor, component of U2 snRNP
TCF4	Transcription factor 4	Helix-loop-helix transcription factor
ZEB2	Zinc finger E-box binding homeobox 2	DNA-binding transcriptional repressor

during OCs (i.e. during neurodevelopment). Genetic variation captured by GWAS may modify the ability of such genes to respond when challenged by ischemia-hypoxia; an oligogenic or polygenic abnormal response could negatively affect the developing brain. A graphic presentation of the G × E concept related to ischemia-hypoxia including the present analyses is provided in **Figure 2**.

While the overall gene set from the most recent GWAS (23) was not enriched for IHR genes, subsets of schizophrenia genes closely related to disease mechanisms were enriched based on a neurodevelopmental model. Genes from schizophrenia GWAS were enriched for mutation-intolerant genes (23), which in turn were strongly enriched for IHR genes. The enrichment of hypoxia-responsive genes among mutation-intolerant schizophrenia genes was replicated with two additional datasets (BH genes, HDB genes). A separate analysis showed that one fifth of genes from schizophrenia GWAS can be linked to monogenic disorders of the nervous system, which were also enriched for IHR genes and BH genes. We propose that the interaction between genetic variants for schizophrenia and effects of ischemia-hypoxia may affect the same cellular functions as the LoF or known mutations, although to a lesser degree. Thus, schizophrenia risk genes with a link to mutations affecting brain development and function provide a specific starting point for functional and experimental studies. In support, it was shown that a set of DD/ASD genes selected based on exome sequencing (29) was also enriched for IHR, BH and HBD genes.

Multiple pathway studies on genomic data in schizophrenia have converged on synaptic mechanisms (25, 26). Using a gene set for synaptic genes [SynGO; ref. (27)], enrichment was confirmed here for the genes in the GWAS loci for schizophrenia. In addition, it was shown with two independent methods that IHR genes are enriched in synaptic genes, indicating the involvement of the synaptic machinery in the response to brain ischemia-hypoxia. Subsequently, an enrichment for IHR and BH genes among the schizophrenia genes related to synaptic functions was detected. This subset of genes is proposed as suitable for functional *in vitro* studies of gene variants under hypoxic conditions.

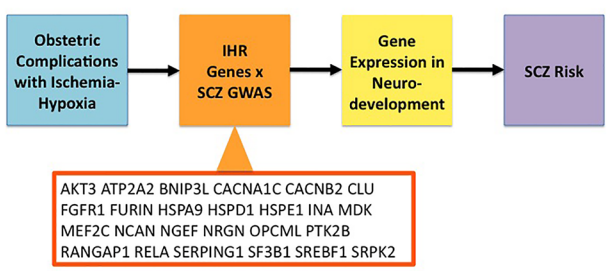

FIGURE 2 | Model for Gene × Environment (G × E) interactions in relation to ischemia-hypoxia and Schizophrenia. Obstetric complications can cause ischemia-hypoxia that activates ischemia-hypoxia response (IHR) genes. A subset of genes from GWAS in schizophrenia (SCZ) overlap with IHR genes as shown here for the genes listed in **Table 1**. Subsequently, ischemia-hypoxia induces changes in gene expression for the overlapping SCZ genes during neurodevelopment. In turn, disturbances of neurodevelopment increase the risk of SCZ. GWAS, genome-wide association study; IHR, ischemia-hypoxia response; SCZ, schizophrenia.

A limitation of the present analysis is that IHR genes are a compilation from several experimental microarray studies of ischemia and hypoxia in animal models. Enrichments for three subsets of schizophrenia genes could be replicated with a large transcriptomics dataset derived by RNAseq in a focal brain ischemia model ["BH genes"; ref. (30)]. The analyses of enrichment for transcriptomics data was based on the assumptions that 18k genes are expressed in the developing brain and that the microarrays and RNAseq provided genome-wide coverage. Responses to ischemia and hypoxia mostly involve changes in transcription ("gene expression") that have been described in multiple, independent experiments. Alterations of pre-mRNA splicing during ischemia-hypoxia also need to be considered (30). Since mRNA stability is also influenced by hypoxia (39), mRNA degradation may variably occur during early stages of ischemic neuronal damage. Genetic variants of schizophrenia risk genes may then influence gene expression, pre-mRNA splicing and/or mRNA stability after ischemia-hypoxia. Abnormal increases and decreases of the expressed proteins can alter neurodevelopmental trajectories, and impairment of protective responses can develop. A caveat is that a set of brain-expressed genes was tested against genomic data for a brain disorder; indeed, recent studies with gene sets obtained for different neuronal populations under physiological conditions showed enrichment with genomic data from schizophrenia (40). The dataset HDB was compiled across several organs on the basis of protein expression (31), and only the link for the subset of LoF genes was significant, but not for the subset for monogenic disorders or synaptic proteins. At this point, it remains unclear whether this finding was related to the use of protein studies which may be less sensitive than mRNA studies.

It remains possible that IHR genes are markers for highly responsive genes in the brain, because ischemia-hypoxia is a very strong stimulus for gene regulation; weaker stimuli such as abnormal neuronal activity could evoke regulation of the same genes that could be relevant for the pathophysiology of schizophrenia.

To address the issue of specificity, HIFs as major regulators of gene expression in response to hypoxia were analyzed (36, 37). The importance of low oxygen levels (relative hypoxia) and HIF regulation in neurodevelopment has been documented in great detail in experimental studies (38). Therefore, we here performed a survey of GWAS-based genes for links to HIF regulation and found several relevant genes, e.g. EP300 (11). Interestingly, a recent combined analysis of gene expression and GWAS data in schizophrenia showed a strong signal for HIF1A in the dorso-lateral prefrontal cortex (41). In the future, it will be of interest to see whether SNPs identified by GWAS in schizophrenia can be aligned with regulatory regions responding to HIFs.

A point of consideration is that our analysis has been focused on neurodevelopment under the assumption that gene x hypoxia

interactions occur in the brain (9). A recent report has shifted the emphasis to gene expression in the placenta in relation to risk of schizophrenia (42). Evidently, the placenta is the central organ for oxygen supply to the embryo and fetus, and ischemic-hypoxic events of the brain during OCs often originate in placental dysfunction. The same gene variants related to ischemia-hypoxia will be expressed in the developing brain and placenta; therefore complex interactions can be envisioned that remain to be addressed. The novel observation that the schizophrenia GWAS dataset contains genes for monogenic disorders of neurodevelopment which overlap with IHR genes supports a role for brain mechanisms. Furthermore, overlap could be also shown for a set of DD/ASD genes which are specifically involved during neurodevelopment.

Vascular factors have been implicated in the emergence of schizophrenia during adolescence and early adulthood (12–14). Abnormalities in lactate levels and brain pH have been measured in manifest schizophrenia (43) which points to abnormalities in glycolytic pathways. Thus, a complementary view could be that the interaction between schizophrenia genes and ischemia-hypoxia (or closely related metabolic perturbations) occurs before or at the onset of psychosis during adolescence. However, direct evidence for vascular dysfunction or overt pathology of cerebral vessels in schizophrenia is presently not available. On the contrary, detailed stereological studies of the capillary system in chronic schizophrenia have not provided evidence for loss of microvessels (44), although larger vessels remain to be studied. It is also conceivable that episodic vascular events become important only on the background of a genetic susceptibility to impaired energy metabolism. In this respect, mitochondrial function has to be integrated into a late-onset model (45, 46).

Finally, the importance of gene variants may only become manifest under conditions of cellular stress (47). Thus, G × E designs using hypoxia are needed to put the hypothesis to the test for schizophrenia-related genes based on GWAS data. Studies capturing multiple medical conditions across the life-span, including detailed pre- and perinatal observations, are needed (48). Even then, the occurrence of ischemia-hypoxia in the fetal brain during OCs has to be assumed, based on experimental studies of maternal-fetal pathophysiology. Expression studies in animal models or cell culture of human neurons may provide new insight. In vivo neonatal models of ischemia-hypoxia have demonstrated regulation of some schizophrenia-related genes (49), but this approach does not capture variation associated with risk of schizophrenia. Studies on developing neurons derived from human iPSCs and exposed to variations in oxygen conditions may shed further light on functional effects of SNPs identified in GWAS for schizophrenia. Interestingly, an initial study using human iPSCs and CRISPR editing has explored a putative causal SNP in FURIN (50) which is an IHR gene and was captured by the present analysis of HIF targets.

A recent study looked for phenotype-specific enrichment of Mendelian disorder genes near GWAS loci across multiple complex traits (51). This provided the rationale for annotating genes in GWAS loci for monogenic (Mendelian) disorders of the nervous system that are known to affect development and function. A broad net was cast by searching for neurodevelopmental and neurological disorders, including disorders of the retina and hearing nerve. Many of these disorders may be traced back to effects of the mutations on early neural development. In the future, it can be tested whether SNPs found in GWAS studies of schizophrenia impact the same developmental process or neuronal function as a known mutation of the same gene.

In conclusion, the analysis of subsets of GWAS-defined schizophrenia genes related to putative disease mechanisms showed significant overlap with gene sets related ischemia-hypoxia, thereby providing cumulative evidence for a role of the hypoxia response in the etiopathogenesis of psychotic disorders. Further studies are warranted to define the genetic risk associated with ischemic-hypoxic events during OCs. Thereby, improved detection of abnormal blood flow and decreased oxygenation in the fetal brain is needed that could lead to novel treatment options.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethics approval and written informed consent was not required as per local legislation and national guidelines.

AUTHOR CONTRIBUTIONS

RS-K wrote the first draft and all authors provided feedback. RS-K generated databases for ischemia-hypoxia response and carried out the enrichment analyses. TK provided expert annotations of HIF genes.

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BRIEF RESEARCH REPORT
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Childhood Trauma Is Nominally Associated With Elevated Cortisol Metabolism in Severe Mental Disorder

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Objective: Individuals exposed to childhood trauma display longstanding modifications of the Hypothalamic–Pituitary–Adrenal (HPA) axis, as well as cognitive impairments. Schizophrenia spectrum disorder (SZ) and bipolar disorders (BD) are characterised by higher prevalence of childhood trauma, abnormal HPA axis, and cognitive dysfunction. Elevated cortisol metabolism was recently demonstrated in both disorders. However, it is yet to be established if childhood adversity is associated with cortisol metabolism in this population, and how this may be associated with cognitive function.

Methods: One-hundred-and-fourteen participants with a DSM-IV SZ or BD diagnosis took part in the study. Diagnoses were evaluated by the Structured Clinical Interview for DSM-IV Axis I disorders (SCID-I). Estimated cortisol metabolizing activity (5α-reductase and 5β-reductase) was assessed by urinary free cortisol, and metabolites. All patients underwent cognitive assessment and completed the Childhood Trauma Questionnaire.

Results: Estimated 5β-reductase activity was elevated in participant with childhood physical abuse ($r = 0.26$, $p = 0.005$). After adjusting for age, sex and diagnosis, physical abuse was still nominally associated with elevated 5β-reductase. Moreover, only high 5α-reductase activity was negatively correlated with working memory and executive performance ($r = -0.23$, $p = 0.01$; $r = -0.19$, $p = 0.05$, respectively), however this disappeared after adjusting for age, sex and diagnosis. Cortisol metabolism did not mediate the association between childhood trauma and cognitive function.

Conclusions: Our study indicates that childhood physical abuse is associated with elevated cortisol metabolism (5β-reductase) in adults with a SZ or BD disorder. However, our study did not support cortisol metabolism as a mediator between childhood trauma experiences and cognitive function within these disorders.

Keywords: childhood trauma and adversity, cognitive function, clinical features, schizophrenia, bipolar disorders, cortisol metabolism

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INTRODUCTION

Individuals exposed to high levels of childhood trauma display long-standing modifications of the biological stress response system, the Hypothalamic–Pituitary–Adrenal (HPA) axis (1). Patients with a schizophrenia (SZ) or a bipolar disorder (BD) report more often childhood traumatic experiences than the general population (2, 3), with HPA axis correlates (1). Both animal (4, 5) and human (6, 7) studies support that early life stress can lead to long-lasting stress-sensitization and dysregulation of the biological stress response. These early life experiences are thought to contribute to the progress of psychosis in susceptible individuals (1). Childhood trauma victims have a heightened negative reaction to distressing experiences later in life (8), elevated cortisol levels over time (9) and blunted responses on stress reactivity tests (10). Both childhood trauma experiences and HPA axis abnormalities are associated with poorer cognitive function in psychotic disorders (1). Nevertheless, to date it is yet to be established if childhood adverse events are also associated with abnormalities in cortisol metabolism in patients with SZ or BD (11) or how this is related to cognitive and clinical features of the disorders.

Studies investigating cortisol metabolism have been performed in Post-Traumatic Stress Disorder (PTSD) (12–14). As discussed by (13), several stress related neuropsychiatric disorders, including PTSD, and chronic fatigue paradoxically exhibit somewhat low levels of cortisol (the biological stress hormone), especially in those traumatized early in life (15), indicating developmental programming and vulnerability to psychopathology (13). In the presence of early life events, the cortisol metabolizing enzymes 5α-reductase and 11β-HSD type 2 activities are reduced (13). Yehuda and colleagues concluded that diminished cortisol metabolism could be a marker of primal susceptibility, potentially by attenuated peripheral catabolism of cortisol resulting in a reduction of the biological stress system sensitivity.

In SZ and BD, two studies by Steen and colleagues concluded with elevated cortisol metabolism (11, 16). Steen et al. reported elevated 5α- and 5β-reductase and 11β-HSD type 2 activity in SZ, whilst BD had intermediate levels between SZ and healthy controls (HC). However, overall 11β-HSD activity was not significantly altered. Elevated cortisol metabolism was proposed as a mechanism for HPA axis dysfunction in these disorders (11, 16, 17). However, the role of childhood trauma within this context is yet to be established. Stressful life events during childhood could lead to HPA axis dysregulation through altered systemic cortisol metabolism (18). Due to the unambiguous findings of the key enzymes 5α- and 5β-reductases in cortisol clearance, these two enzymes are of specific interest. They constitute the pathway with the most consistent finding of increased cortisol clearance in SZ and BD, and they are key enzymes in cortisol metabolism catalyzing irreversible conversion of cortisol and are both expressed in the liver. Moreover, cortisol has been shown to have an impact on cognition in in BD and SZ (1). However, it is yet unknown if poorer cognitive function evident in individuals with childhood trauma experiences (19, 20) associated with cortisol metabolism.

Our hypotheses are as follows: In patients with SZ and BD, childhood trauma and cognitive function will be related to cortisol metabolism activity, and cortisol metabolism will mediate the association between childhood trauma and cognitive function. We focused on the important rate-limiting enzymes 5α- and 5β-reductases as both consistently indicate increased hepatic cortisol clearance in SZ and BD.

METHODS

Subjects

Participants were recruited consecutively from outpatient and inpatient units from four hospitals in Oslo as part of the larger NORMENT Research study. A sub-sample of subjects included from 2006 to 2010 had their urine sampled for estimation of systemic cortisol metabolizing activity. Enzyme activities were not estimated for individuals with diagnoses of hepatic- or renal disorder, thyroid dysfunction, or use of corticosteroid medications (11). The sub-sample included participants with information on urinary cortisol metabolites, childhood trauma variables from the CTQ screening interview and a standardized extensive cognitive battery, consisting of a total of 114 patients (63 schizophrenia spectrum disorder, SZ [34 schizophrenia; six schizophreniform disorder; nine schizoaffective disorder; 14 other psychosis]; 47 bipolar disorder, BD [32 bipolar I; six bipolar NOS; nine bipolar II]; and four major depressive disorder with psychotic features). The sample is part of larger sample of cortisol metabolism data previously reported on by Steen et al. (11, 16) the current sub-sample also had recording of childhood trauma variables.

Exclusion criteria for all groups were: poor fluency in Norwegian language or organic psychosis, or IQ below 70. The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate approved the study. Written informed consent were given by all participants.

Clinical Assessment

Clinical assessments were carried out by qualified clinical trained doctors, psychiatrists and clinical psychologists. The structured Clinical Interview for DSM-IV Axis I disorders (SCID-I) was used to assess diagnostic criteria's of SZ and BD. Diagnostic reliability was found acceptable with overall agreement for DSM-IV diagnostic categories of 82% and the overall κ 0.77 (95% CI: 0.60–0.94). Positive and negative symptoms during the last seven days were assessed by the Positive and Negative Symptom Scale (PANSS) (21). Inter-rater reliability was satisfactory with intra-class correlation coefficients for PANSS subscales in the range of 0.71 to 0.73 (22). Function level was assessed using a split version of the Global Assessment of Functioning Scale [GAF; (23)].

Cognitive Assessment

Cognitive assessment was performed by psychologists trained in standardized neuropsychological test batteries. The neuropsychological test battery was administered in a fixed order and took 3-hour with two breaks with refreshments. In

this study, we specifically focused on measures sensitive to dysfunction in SZ and BD, and to stress (24–26). Five cognitive areas were assessed: 1) Verbal learning and memory; 2) Working memory 3) Executive function 4) Performance intelligence and 5) Verbal intelligence. Standardized z-scores were calculated based on healthy controls performance (mean and standard deviation) and collapsed into domain scores. The following neurocognitive domains were calculated:

Learning and Memory was assessed using the California Verbal Learning Test (CVLT) II, including sub items for learning, delayed recall and recognition (27). *Working memory* was assessed using Letter-Number Sequencing and Digit Span (WAIS-III) (28). **Ex***ecutive functioning* was assessed with the Verbal Fluency Test (Delis–Kaplan Executive Function Scale (D-KEFS) (27) with measures of phonetic fluency and semantic fluency. *Performance abilities* were measured using Block Design and Matrix Reasoning from the Wechsler Abbreviated Scale of Intelligence (WASI) (29). *Verbal abilities* were measured using Similarities and Vocabulary from WASI (29). All cognitive scores were presented as z-scores constructed based on the overall baseline mean and standard deviation from the healthy controls (healthy controls IQ score 113 ± 10.04; range 78–138) in the larger TOP sample [for more details see (30)].

Childhood Trauma Questionnaire (CTQ)

The Childhood Trauma Questionnaire (CTQ) was applied to assess for traumatic events in childhood (see (30, 31), including emotional abuse (EA), physical abuse (PA), sexual abuse (SA), physical neglect (PN), and emotional neglect (EN). CTQ is a self-report questionnaire and each subscale is comprised of five items rated on a 5-point Likert scale ranging from 1 (never true) to 5 (very often true). For more detailed please see (30, 32).

Design and Cortisol Measurements

All patients took part in the cognitive testing and routine blood withdrawal, and spot urine was sampled for analyses of urinary free cortisol (UFF), urinary free cortisone (UFE), allo-tetrahydrocortisol (aTHF), tetrahydrocortisol (THF) and tetrahydrocortisone (THE) (11). The Institute of internal medicine, University of Bergen and the Hormone Laboratory, Haukeland University Hospital performed the measurements of allo-THF, THF, THE, UFF and UFE based on liquid chromatography tandem mass spectrometry (LCMSMS) (for details see (17). Indexes of enzyme activities: Activities of the 5α- and 5β reductases were calculated with aTHF/UFF and THF/UFF indexes respectively (33). Urinary creatinine was measured (Jaffé-reaction, Cobas Integra, Roche Diagnostics GmbH, Mannheim, Germany) to adjust for urine concentration.

Statistical Analyses

Data were analyzed using IBM SPSS Statistics, Version 26.0. Spearman's correlations were performed for the bivariate associations between childhood trauma, cortisol-metabolizing enzyme activities and cognitive domains. Independent sample t-tests were applied comparing enzyme activities of participants on regular treatment with at least one drug regularly of either antipsychotics, antidepressants or mood stabilizers to participants not on regular treatment with these pharmacological agents. Further analyses subdividing into number of drugs within each drug class were conducted using analysis of variance (ANOVA). Data were log transformed before analyzed by independent sample t-tests, ANOVA or multiple regression analyses. Hayes mediation model (34) was applied to investigate if cortisol metabolism mediated the relationship between a history of childhood trauma experiences and poorer cognitive functioning with built in bootstrapping. The mean and median time of the samples was 11 am. As no significant associations were observed for BMI levels, urine concentration and enzyme activities or ($p > 0.1$), we did not adjust the results for BMI levels, or urine concentration. Regression models (including the mediation models) were adjusted for age, sex and diagnosis. Similar to (30), the four participants with MDD with psychotic features were added to the BD group to create an "affective group". To rule out type 1 error, we adjusted for analyzing several cognitive domains and childhood trauma subtypes, applying a significance level of 0.01 instead of <0.05. As this was a hypothesis driven study, we decided this was appropriate without the possibility of losing vital information.

RESULTS

Overview of the Sample

In the total sample, the mean age at inclusion was 32 years, and 60% were males. Mean years of education was thirteen. Patients with SZ were younger and had fewer years of education than patients with BD. Patients with SZ had more severe symptoms from the PANSS and poorer functioning from the GAF and scored poorer on all cognitive tests compared to the BD group. Patients with SZ also had higher 5α-reductase activity than BD, whilst contrary to the larger sample (11) no statistically significant association were observed between groups for 5β-reductases. There were no differences in childhood trauma experiences between SZ and BD (see **Table 1**). N = 82 (72%) of the patients were regularly prescribed antipsychotic medication, 43 (38%) were regularly receiving antidepressants and 31 (27%) were receiving anticonvulsants or lithium (mood stabilizers). No association was observed for enzyme activities comparing users and non-users of antipsychotics (independent sample t-test, 5α-reductase activity, $t = 0.18$, $p = 0.86$; 5β reductases, $t = 0.32$, $p = 0.75$), users and non-users of antidepressants (5α-reductase activity, $t = -1.02$, $p = 0.31$; 5β reductases, $t = -0.83$, $p = 0.41$), or of mood stabilizers (5α-reductase activity, $t = -1.58$, $p = 0.12$; 5β reductases, $t = -0.21$, $p = 0.84$). Further analysis within each drug class showed that 32 (28%) were not regularly prescribed antipsychotics, 67 (59%) were receiving one type of antipsychotic medication, and 15 (13%) were receiving at least two types of antipsychotics. No association was observed between the groups and cortisol metabolism (ANOVA, 5α-eductases: $F = 0.25$, $p = 0.78$; 5β-reductases, $F = 0.53$, $p = 0.59$). However, the current study is a subsample of Steen et al. (11) where analyses showed a nominal increase in 5β-reductase with use of antipsychotics. Seventy-one (62%) were not prescribed any regular antidepressants, 28 (25%) were receiving one type of antidepressant, nibe (8%) were receiving two

TABLE 1 | Demographics of the patients and clinical characteristics divided into schizophrenia and bipolar disorders.

	SZ N = 63	BD N = 51	Statistics
Age (mean ± SD)	28.9 ± 8.9	35.9 ± 12.2	F = 12.5, DF = 1, P = 0.001
Sex (M/F)	37/26	23/28	X^2 = 2.1, DF = 1, P = 0.15
Years of education (mean ± SD)	12.2 ± 2.8	14.3 ± 3.6	F = 12.6, DF = 1, P = 0.001
GAF (mean ± SD)	44.3 ± 11.1	49.9 ± 10.9	F = 7.4, DF = 1, P = 0.007
PANSS total score	63.6 ± 20.1	45.7 ± 10.7	F = 32.7, DF = 1, P < 0.001
Childhood trauma total score (mean ± SD)	46.6 ± 15.9	45.1 ± 19.9	F = 0.28, DF = 1, P = 0.60
Physical Abuse, (mean ± SD)	7.6 ± 3.8	7.4 ± 4.4	F = 0.08, DF = 1, P = 0.79
Sexual Abuse (mean ± SD)	7.3 ± 4.4	7.0 ± 4.8	F = 0.15, DF = 1, P = 0.70
Emotional Abuse (mean ± SD)	11.1 ± 4.7	10.1 ± 5.4	F = 1.13, DF = 1, P = 0.29
Physical Neglect (mean ± SD)	12.8 ± 5.1	12.6 ± 5.4	F = 0.05, DF = 1, P = 0.83
Emotional Neglect (mean ± SD)	8.3 ± 3.1	7.5 ± 3.8	F = 1.22, DF = 1, P = 0.27
Memory (mean ± SD)[a]	–0.9 ± 1.4	–3.1 ± 1.2	F = 5.20, DF= 1, P = 0.02
Working memory (mean ± SD)[a]	–0.8 ± 0.8	–0.4 ± 0.9	F = 11.1, DF = 1, P = 0.001
Executive function (mean ± SD)[a]	–1.3 ± 1.1	–0.5 ± 1.2	F = 6.0, DF = 1, P = 0.02
Verbal abilities (mean ± SD)[a]	–1.3 ± 1.5	–0.6 ± 1.1	F = 7.9, DF = 1, P = 0.006
Perception and visuo-spatial abilities (mean ± SD)[a]	–1.1 ± 1.6	–0.6 ± 1.4	F = 3.3, DF = 1, P = 0.07
5β-reductase activity (mean ± SD)	96.9 ± 58.4	82.4 ± 56.3	F = 2.3, DF = 1, P = 0.13
5α-reductase activity (mean ± SD)	112.2 ± 103.3	75.3 ± 58.2	F = 6.3, DF = 1, P = 0.01

SZ, Schizophrenia; BD, bipolar disorder. 99.1% (n = 113) of the patients with estimated cortisol metabolism activity completed physical abuse subscale; 97.4% (n = 111) completed sexual abuse subscale; 98.2% (n = 112) completed emotional abuse subscale; 99.1% (n = 113) completed emotional neglect subscale, and 97.4% (n = 111) completed physical neglect subscale. 96.5% (n = 110) had complete data on years of education. a = z score.

types of antidepressants and six (5%) were receiving three or more types of antidepressants. No association was observed between groups and cortisol metabolism (ANOVA, 5α-eductases: F = 1.97, p = 0.12; 5β-reductases, F = 0.88, p = 0.45). As only one person was prescribed more than one type of mood stabilizer, only antipsychotics and antidepressants were broken down to number of different drugs.

Cortisol Metabolism and Childhood Trauma

5β-reductase was positively correlated with physical abuse experiences (Spearman's correlation [rho], r = 0.26; p = 0.005), moreover, correlations with sexual abuse (r = 0.20, p = 0.04), and emotional abuse (r = 0.17, p = 0.07) were indicated (see **Table 2**). 5α-reductase was positively correlated at trend level with emotional neglect (r = 0.18, p = 0.06). After correcting for confounders (age, sex and diagnosis) physical abuse was still nominally associated with higher 5β reductases (see **Table 3**).

Cortisol Metabolism, Cognitive Function and Clinical Characteristics

5α-reductases were negatively correlated with working memory (Spearman's correlation [rho], r = −0.23, p = 0.01). An association with executive function (r = −0.19, p = 0.05) was also suggested. However, neither the association with working memory nor with executive function was statistically significant or at trend level after adjusting for age, sex and diagnosis (B = −0.13 SE = 0.09, t = −1.29, p = 0.21; B = −0.08, SE = 0.06, t = −0.75, p = 0.45, respectively). Similarly, 5α-reductases was positively correlated with total PANSS score (as a measure of more severe current symptoms, r = 0.23, p = 0.01) and negatively correlated at a nominal level with GAF-F (with lower GAF-F as a measure of poorer functioning, r = 0.22, p = 0.02). After adjusting for age, sex and diagnosis there were no longer a relationship between 5α-reductases and GAF-F or 5α-reductases and total PANSS score (B = −0.13, SE = 0.007, t = −1.34, p = 0.18; B = 0.08, SE = 0.004 t = 0.75, p = 0.45, respectively). 5β-reductase was not associated with the cognitive or clinical measures.

TABLE 2 | Association between cortisol metabolism, childhood trauma and cognitive function.

	5β reductases (n = 114)	5α Reductases (n = 114)
Physical abuse	r = 0.26 p = 0.005	r = 0.15 p = 0.10
Sexual abuse	r = 0.20 p = 0.035	r=0.12 p=0.22
Emotional abuse	r = 0.17 p = 0.07	r=0.12 p=0.19
Physical neglect	r = 0.07 p = 0.49	r = 0.06 p = 0.57
Emotional neglect	r = 0.12 p = 0.20	r = 0.18 p = 0.06
Memory	r = 0.05 p = 0.60	r = –0.02 p = 0.80
Working memory	r = –0.15 p = 0.11	r = –0.23 p = 0.01
Executive function	r = –0.15 p = 0.11	r = –0.19 p = 0.05
Verbal abilities	r = 0.04 p = 0.70	r = –0.10 p = 0.30
Perception and visuo-spatial abilities	r = –0.06 p = 0.53	r = –0.09 p = 0.34
GAF	r = –0.17 p = 0.08	r = –0.22 p = 0.02
PANSS	r = 0.14 p = 0.15	r = 0.23 p = 0.01

Spearman's correlation; aTHF, allo-tetrahydrocortisol; THF, tetrahydrocortisol; THE, tetrahydrocortisone; UFF, urinary free cortisol; UFE, urinary free cortisone; 5α- and 5β reductases were calculated with aTHF/UFF and THF/UFF indexes respectively.

TABLE 3 | Linear regression with possible confounders of the correlation between 5β reductases and physical abuse.

	B	SE	T	p value
Constant	4.05	0.26	15.47	˂0.001
Age	0.01	0.01	2.20	0.03
Sex	0.03	0.11	0.27	0.79
Diagnosis (SZ/BD)	–0.24	0.12	–2.05	0.04
Physical abuse	0.03	0.01	2.09	0.039

SZ, schizophrenia, BD, Bipolar disorders.

Lastly, *process* confirmed that cortisol metabolism (5α-reductase or 5β-reductase) did not mediate the association between childhood physical abuse and cognitive function (working memory or executive functioning, p >0.1; see adjusted values in **Supplementary Material Figures S1–4**). Based on the negative findings in **Table 2**, no other mediation analyses were ran.

DISCUSSION

Our study indicates that childhood adverse events are associated with elevated cortisol metabolism (5β-reductase) in adults with a SZ or BD disorder. After adjusting for potential confounders, this was only suggested for 5β-reductases and physical abuse. Cortisol metabolism may affect HPA axis activity (35), and is suggested as a factor for HPA axis dysfunction in SZ and BD (11, 16). Individuals with extreme stressful experiences in childhood, including PTSD have changes in their cortisol metabolism profile (13, 36). However prior to our study this had not yet been investigated in SZ or BD, even though patients with SZ and BD more often report childhood trauma experiences than individuals without a mental disorder (2).

Individuals with childhood trauma experiences have attenuated cortisol metabolism than those without trauma (13). Contrary to what observed in PTSD, a history of childhood trauma was accompanied by elevated cortisol metabolism in SZ and BD, specifically in individuals who also reported childhood physical abuse. These findings support a distinct cortisol metabolism profile in SZ and BD affected by childhood adverse experiences. Differences in cortisol metabolism between the diagnostic groups are supported by altered levels of cortisol in in PTSD compared to SZ or BD, with low cortisol levels reported in PTSD (13), while elevated diurnal cortisol levels are often reported in SZ or BD (9, 37, 38). Similarly, in major depression there are indications of reduced cortisol clearance (39, 40).

The suggested link between poorer cognitive function (working memory, executive functioning), and poorer clinical functioning (GAF and PANSS) and elevated cortisol metabolism (5α-reductases) was no longer statistically significant or at trend level after adjusting for potential confounders, such as age, sex and diagnosis. As discussed in Pruessner et al. (1), the HPA axis is vital for survival as it allows the organism to prepare for hostile events and to recover after stress experiences. On the other side, long-term activation of the biological stress system can have unfavorable effects on brain structure/function and behavior (41, 42). High levels of stress over time have been linked to changes in regional brain volumes and neuronal structure and function. In fact, severe and chronic stress, both in childhood and in adulthood, can cause neural cell death and atrophy of neuronal processes, and influence hippocampus neurogenesis and plasticity (43–45). Our negative findings suggest that sampling techniques focusing on long-term exposure of cortisol (such as cortisol in hair) may be a more robust marker to capture stress related brain changes within these groups than a one-time point sample targeting transitory cortisol levels, which can be affected by diurnal rhythm, activity levels and the day-night cycle (9).

This study had several strengths: The well-characterized large sample size with psychological assessment as well as biological markers (here 5α-reductase and 5β-reductase activity), and information on trauma history. Moreover, this study covers an important gap in the literature addressing cortisol metabolism and childhood trauma in SZ and BD. We focused our study on important rate limiting enzymes catalyzing irreversible conversions and showing consistent alterations in previous studies (11, 16).

Some limitations of the study should be mentioned: Firstly, within this cohort we did not have data on childhood trauma experiences for the healthy controls, thus we were unable to investigate the role of childhood trauma experiences on cortisol metabolism in a healthy population. However numerous of studies show that patients with SZ and BD report more childhood trauma experiences than HC (1, 2, 46), suggesting vulnerability for alteration in cortisol metabolism in patients with SZ or BD due to higher trauma exposure. Furthermore, information about childhood trauma was assessed in adults using the Childhood Trauma Questionnaire (CTQ), a retrospective questionnaire about trauma experiences in childhood. A recent meta-analysis study found low overlap between retrospective and prospective data on childhood trauma (47), however large heterogeneity was reported across studies. Encouragingly, convergent validity of prospective and retrospective data has been suggested as they are associated with similar outcomes (48), supporting the use of retreospective design. Although our finding of an association between higher cortisol metabolism and reports of physical abuse were independent of age, sex, and diagnoses, we cannot rule out that other confounding factors may be present that we have not adjusted for. Although we adjusted for number of trauma subtypes and number of cognitive tests using a more stringent P value of 0.01 than the standard of less than 0.05, we cannot rule out potential spurious findings. Lastly, after adjusting for potential confounders we no longer found an association between cortisol metabolism and cognitive (working memory, executive functioning) or clinical features (GAF, PANSS). However, we cannot rule out the presence of subgroups (clusters) within the sample. For example, it could be subgroups of individuals characterized by high levels of

trauma, elevated cortisol metabolism and poorer cognitive function, and other clusters characterized by high load of genetic vulnerability, poor cognition and no history of childhood trauma. Due to our relatively small sample (n = 114) we were not able to perform these additional cluster analyses.

To conclude, our study indicates that physical abuse in childhood is associated with elevated cortisol metabolism (5β-reductase) in adults with a SZ or BD disorder, demonstrating a distinct cortisol metabolism profile in SZ and BD affected by childhood adverse experiences. Whether cortisol metabolism is associated with cognitive and clinical characteristics within subgroups of these disorders needs to be further investigated for example by applying cluster analysis in larger samples.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors (MA, TU, AI, IM, OA, NS) listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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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.2020.00391/full#supplementary-material

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The Long-Term Effects of Early Life Stress on the Modulation of miR-19 Levels

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MicroRNAs (miRNAs), one of the major small non-coding RNA classes, have been proposed as regulatory molecules in neurodevelopment and stress response. Although alterations in miRNAs profiles have been implicated in several psychiatric and neurodevelopmental disorders, the contribution of individual miRNAs in brain development and function is still unknown. Recent studies have identified miR-19 as a key regulator of brain trajectories, since it drives the differentiation of neural stem cells into mature neurons. However, no findings are available on how vulnerability factors for these disorders, such as early life stress (ELS), can modulate the expression of miR-19 and its target genes. To reach our aim, we investigated miR-19 modulation in human hippocampal progenitor stem cells (HPCs) treated with cortisol during 3 days of proliferation and harvested immediately after the end of the treatment or after 20 days of differentiation into mature neurons. We also analyzed the long-term expression changes of miR-19 and of its validated target genes, involved in neurodevelopment and inflammation, in the hippocampus of adult rats exposed or not to prenatal stress (PNS). Interestingly, we observed a significant downregulation of miR-19 levels both in proliferating (FC = −1.59, p-value = 0.022 for miR-19a; FC = −1.79, p-value = 0.016 for miR-19b) as well as differentiated HPCs (FC = −1.28, p-value = 0.065 for miR-19a; FC = −1.75, p-value = 0.047 for miR-19b) treated with cortisol. Similarly, we found a long-term decrease of miR-19 levels in the hippocampus of adult PNS rats (FC = −1.35, p-value = 0.025 for miR-19a; FC = −1.43, p-value = 0.032 for miR-19b). Among all the validated target genes, we observed a significant increase of NRCAM (FC = 1.20, p-value = 0.027), IL4R (FC = 1.26, p-value = 0.046), and RAPGEF2 (FC = 1.23, p-value = 0.020).We suggest that ELS can cause a long-term downregulation of miR-19 levels, which may be responsible of alterations in neurodevelopmental pathways and in immune/inflammatory processes, leading to an enhanced risk for mental disorders later in life. Intervention strategies targeting miR-19 may prevent alterations in these pathways, reducing the ELS-related effects.

Keywords: early life stress, miR-19, brain trajectories, neurodevelopment, inflammation, depression, schizophrenia

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INTRODUCTION

MicroRNAs (miRNAs) represent one of the major small non-coding RNA classes that have been proposed as regulatory molecules in several biological processes, including neurodevelopment and stress response (1–4). They are critical regulators of gene expression and exert their activity through the modulation of target mRNA stability or translation efficiency. Indeed, the miRNA binding, primarily to the 3′UTR of mRNAs, leads to mRNA destabilization or translational repression, resulting in reduced protein levels of the miRNA-target genes (5). Therefore, miRNAs have been also named as "master regulators", because one miRNA can regulate hundreds of genes within a specific biological or cellular pathway and more than half of the protein-coding genes are predicted to be regulated by miRNAs (6).

Alterations in miRNAs profiles have been implicated in several psychiatric and neurodevelopmental disorders, as Major Depressive Disorder (MDD) (7–9) and Schizophrenia (SZ) (10–13), two complex and severe diseases characterized by dysregulation of behavior, emotion, and cognition (14). Although the potential role of epigenetic deregulation in the pathogenesis of psychiatric disorders is a major focus of the current research (15, 16), the contribution of individual miRNAs in brain development and function and, consequently, in the pathophysiology of psychiatric illnesses is still largely unknown.

Epidemiological and experimental findings indicate that early life adversities occurring during the pre, peri and postnatal period can increase the vulnerability of developing psychiatric disorders later in life (17–19). Indeed, different environmental factors, including exposure to maternal stress, psychiatric disorders, alcohol, drugs, chemicals, poor nutrition, and infections during pregnancy have a negative impact on brain development during fetal and postnatal life and they have been indeed associated with the future onset of psychiatric disorders or altered behaviours in the offspring (20). Moreover, childhood trauma is a well-known clinical risk factor for the development of psychopathology later in life in the exposed individuals (21–23). For instance, several meta-analyses have provided robust evidence for an association between childhood trauma and SZ (24, 25). For example, a longitudinal 10-year prospective cohort study of 3,021 adolescents and young adults showed that experiences of childhood trauma and recent life events (namely, the second challenge) are strongly correlated and interact additively in increasing the risk for psychosis (26). Similarly, in another prospective study, a large cohort of adolescents and adults who have been sexually abused before the age of 16 years old showed a 2-fold increased risk for a psychotic disorder and a 2.6-fold increased risk for SZ (27).

Recent studies have identified miR-19 as a key regulator of neurodevelopmental brain trajectories, since it drives the differentiation of neural stem cells into mature neurons (28–31). MiR-19, a family composed of miR-19a and miR-19b that differ from each other by one single nucleotide in the middle of the sequence is a member of a polycistronic miRNA gene, namely, the miR-17/92 cluster. This cluster includes miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a and is mainly involved in brain development and function. In humans, a microdeletion of this cluster results in the Feingold syndrome type II characterized by microcephaly, mild intellectual disabilities, and psychiatric symptoms, including MDD and anxiety (32–34). Conversely, a microduplication of the genomic locus including miR-17/92 results in mild macrocephaly and autism spectrum disorder (35). Several studies have also established that the miR-17/92 cluster regulates the proliferation and oligodendrogenesis of neural progenitor cells (NPCs) during brain development (30, 36, 37). In this context, Bian and collaborators have recently demonstrated that miR-19 exerts a key role in the developing mouse neocortex, promoting neural stem cell proliferation, and modulating the NPCs fate, through the repression of phosphatase and tensin homolog (PTEN) protein (30). Similarly, Han and collaborators have revealed that an over-expression of miR-19 in adult hippocampal NPCs facilitates cell migration and newborn neuron deposition in the adult brain by directly targeting Rap Guanine Nucleotide Exchange Factor 2 (RAPGEF2), a member of the Rapgef family known to be involved in neuronal migration (38). These data confirm the involvement of miR-19 in neurodevelopment, above all in the regulation of the first steps of adult neurogenesis. Moreover, by using human hippocampal NPCs derived from adult SZ patients (SZ-NPCs) and relative controls, the same authors (29) found that miR-19 expression levels were higher in SZ-NPCs as compared to those obtained from controls. This finding is consistent with a previous study reporting increased levels of miR-19 in post-mortem brains of SZ patients (39) and suggests this miRNA as a molecule associated to SZ, with the potential to affect brain connections and functions.

Although the above-mentioned studies have demonstrated that alterations in miR-19 expression are associated with an aberrant neurodevelopment or with a specific clinical feature, none of these studies has focused the attention on the effects of early life stress (ELS), one of the most important clinical risk factor for mental disorders, which negatively affects brain developmental trajectories, in modulating miR-19 levels.

Therefore, considering that stressful experiences in sensitive periods of life, when brain is still under maturation, can influence the correct processes of neurodevelopment (40–43) as well as neurogenesis (44), in this study, we aimed to investigate whether ELS can modulate miR-19 expression levels and its related pathways and whether its long-lasting effects persisted overtime. In our previous paper (4), we performed miRNomics analyses in different tissues and species all mimicking a condition of early stress, and, among the significantly modulated miRNAs, we identified miR-19a-3p, which was downregulated in proliferating HPCs treated with cortisol, and miR-19b-3p, which was downregulated as well in the hippocampus of adult prenatal stress (PNS) exposed rats as compared to control animals. Based on these findings, in the current work, we decided to follow up our results on this miRNA, examining more in details the short and long-lasting effects of ELS on miR-19 expression levels and also investigating the miR-19 variants represented by miR-19a and miR-19b. Moreover, we have also

measured a panel of miR-19 validated target genes involved in neurodevelopment and inflammation, to evaluate the role of miR-19 in the modulation of these biological pathways.

We tested our hypothesis by measuring the short and long-term modulation of miR-19a and miR-19b in our well-established *in vitro* model of stress represented by HPCs treated with cortisol, the stress hormone, during 3 days of proliferation or after 20 days of differentiation into mature neurons without any treatment. We also measured miR-19a and miR-19b in the hippocampus of adult rats exposed *in utero* to PNS as compared to control animals.

MATERIALS AND METHODS

Cell Culture

The immortalized multipotent, human hippocampal progenitor cell line, HPC0A07/03C (propriety of ReNeuron), was used for all the experiments. Because of these cells are grown in a tightly controlled experimental environment, this model allows overcoming the unavoidable variability of clinical samples and to reproduce data from human brain cells, inaccessible in patients (44). As previously described (4, 45), HPC0A07/03C cells proliferate indefinitely in the presence of epidermal growth factor (EGF), fibroblast growth factor (bFGF), and 4-hydroxytamoxifen (4-OHT), whereas differentiation is induced by the removal of these molecules.

We performed two different treatments. **Treatment 1 (short-term effects):** HPC0A07/03C cells were treated for 3 days during proliferation with cortisol (100 μM) or vehicle and cells were harvested immediately after the end of the treatment. **Treatment 2 (long-term effects):** HPC0A07/03C cells were treated with cortisol (100 μM) or vehicle during 3 days of proliferation and harvested after 20 days of differentiation into mature neurons without any treatment. Five biological replicates were obtained for each treatment and condition (cortisol or vehicle).

By using these *in vitro* paradigms, we aimed to identify changes in miR-19 expression levels as consequence of the direct exposure to stress during development (mimicked by cortisol treatment in the proliferation phase, namely, Treatment 1) and to evaluate whether such changes could be maintained over time, after differentiation into mature neurons (Treatment 2).

PNS Model

The PNS paradigm was performed as already published (4, 40, 46). Briefly, pregnant dams were restrained in a transparent Plexiglas cylinder, under bright light, for 45 min, three times a day during their last week of gestation. Control pregnant females were left undisturbed in their home cages. Adult male offspring from the control and the PNS groups was sacrificed at postnatal day (PND) 62 for the whole dissection of the hippocampus. Rat handling and experimental procedures were performed according to the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (D.L. 116/92), in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. RNA samples from the hippocampus of animals exposed or not to PNS (n = 10 per group) at PND 62 were used for the expression analyses of miR-19a, miR-19b, and its target genes.

RNA Isolation

Total RNA, including miRNAs, was isolated from HPC0A07/03C cells using the AllPrep DNA/RNA/miRNA kit (Qiagen, Hilden, Germany) and from rats' brains using PureZol RNA isolation reagents (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocols. RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Real-Time PCR

The expression levels of miR-19 were analyzed in proliferating or differentiated HPC0A07/03C cells (treated with cortisol 100 μM or vehicle) by Real-Time PCR using the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA) and the TaqMan MicroRNA Assays (Thermofisher, Waltham, MA, USA), following the manufacturer's instructions.

MiR-19 includes miR-19a and miR-19b, which differ from each other by one single nucleotide in the middle of the sequence, so we measured both of them in our experimental model (for details, see https://www.thermofisher.com/order/genome-database/details/mirna/000395 and https://www.thermofisher.com/order/genome-database/details/mirna/000396). In order to analyze the modulation of miR-19a and miR-19b, a total amount of 50 ng of the extracted total RNA, including miRNAs, from each sample was firstly reverse transcribed using the TaqMan MicroRNA RT Kit (Thermofisher, Waltham, MA, USA) and, subsequently, the RT product was pre-amplificated using TaqMan PreAmp Master Mix (Thermofisher, Waltham, MA, US). At this point, the PreAmp product was diluted with 0.1X TE and then evaluated for the expression levels analysis of miR-19a and miR-19b by Real-Time PCR using the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA), the TaqMan MicroRNA Assays (Thermofisher, Waltham, MA, USA) and the TaqMan Universal Master Mix, no AmpErase UNG (Thermofisher, Waltham, MA, USA) following the manufacturer's instructions. The relative expression of miR-19a and miR-19b was normalized to the levels of the housekeeping gene RNU44 in HPC0A07/03C cells (47, 48) and to the levels of U6 in rats. All the reactions were performed in triplicates.

The expression levels of several miR-19 validated target genes, such as Phosphatase And Tensin Homolog (PTEN), Phosphoinositide-3-Kinase Regulatory Subunit 1 (PIK3R1), Adrenoceptor Beta 1 (ADRB1), Neuronal Cell Adhesion Molecule (NRCAM), Transforming Growth Factor Beta Receptor 2 (TGFBR2), RAPGEF2, Tumor Necrosis Factor alpha (TNFα), Interleukin 4 Receptor (IL4R), and the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were evaluated in the hippocampus of adult PNS rats by using TaqMan Assays (Thermofisher, Waltham, MA, USA) on the

CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions.

The Pfaffl Method was used to determine the relative expression values of miR-19a, miR-19b and of genes of interest (49).

Gene-Targeting Prediction and Validation Analyses

The gene-targeting analyses for predicted and validated target genes of miR-19a and miR-19b were performed by using miRWalk2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (50), a comprehensive database that provides predicted as well as validated miRNA binding site information. To make any possible inference and to obtain a comparative view, miRWalk automatically combines and integrates the identified miRNA binding sites with the results obtained from other established miRNA-target prediction databases (miRWalk, miRanda, and Targetscan). Specifically, we used the sequences, annotation and accession number available in the miRBase database (http://www.mirbase.org/) (51), such as MI0000073 for mir-19a, MI0000074 for mir-19b-1, and MI0000075 for mir-19b-2.

In Silico Analysis of Candidate Genes

In order to evaluate the potential involvement of differentially regulated genes in association with the effects of exposure to stress early in life, we considered open target genetic prioritization platform (https://genetics.opentargets.org/). This platform performs an integrative analysis including the evaluation of genetic signal from genome-wide association study (GWAS) Catalog and UK Biobank as well as databases annotation from multiplex public available resources (genetic association, expression, literature, animal models). By integrating different kind of data from omics data to automatic information retrieval from literature, the open target platform is capable to visualize target gene-disease associations (52). The combination of GWAS and functional genomics data have allowed to prioritise likely causal variants at disease-associated loci (53).

Moreover, we performed a Transcriptome Wide Association Study (TWAS) for MDD and SZ in human considering brain tissues and blood (http://twas-hub.org/). These models integrate genome wide associations for a given phenotype (i.e., MDD or SZ) with expression quantitative loci (eQTL) from gene-expression tissue database, such as GTEx (https://gtexportal.org/home/) and common mind consortium (https://www.nimhgenetics.org/resources/commonmind) to test whether or not a differential tissue-specific gene expression regulation can be expected according to the genetic and the eQTL signal. By mean of TWAS, it is possible to prioritize gene associations starting from GWAS by estimating gene-expression downregulation and upregulation in a tissue specific manner. In fact, many genetics variants influence complex trait by modulating gene expression. Specifically, TWAS quantify the association between the genetically regulated levels of gene expression and the phenotype by mean of different regression models (54).

Pathway and Network Analyses

Lists of validated target genes of miR-19a, miR-19b-1, and miR-19b-2 were imported into Ingenuity Pathway Analyses Software (IPA) for pathway and network analyses. The "Core Analysis" function included in IPA (Ingenuity System Inc., USA http://www.ingenuity.com) was used to understand and discuss the data in the context of biological processes, pathways and networks associated with the experimental system. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/pathway.html) was used to investigate the molecular interaction, reaction, and relation networks among the biological systems.

Statistical Analyses

Statistical analyses were conducted using SPSS version 24.0 statistical software (SPSS Inc., Chicago, IL, USA) and data are expressed using box-plot. For comparison of variables between groups, Student's t-test was applied.

RESULTS

Effect of ELS on miR-19a and miR-19b Expression Levels in the In Vitro Model

With the aim to investigate the effects of ELS on the modulation of miR-19a and miR-19b, we used our well-established *in vitro* model of stress represented by the HPC0A07/03C cells that we treated with the stress hormone cortisol 100 μM (20, 40, 45). First, we looked at miR-19a and miR-19b expression levels immediately after 3 days of cortisol treatment and we observed a statistically significant down-regulation of both miRNAs in proliferating cells as compared to vehicle (FC = −1.59, p-value = 0.022 for miR-19a; FC = −1.79, p-value = 0.016 for miR-19b) (**Figure 1A**). Interestingly, after 20 days of differentiation without any treatment, we found a similar trend of decrease of miR-19a (FC = −1.28, p-value = 0.065) and a significant downregulation of miR-19b expression levels (FC = −1.75, p-value = 0.047) in mature neurons, which were previously sensitized by cortisol treatment during 3 days of proliferation as compared to vehicle (**Figure 1B**).

These *in vitro* results suggest that an exposure to high concentration of cortisol affects the short-term modulation of miR-19a and miR-19b with long-lasting effects that can be also observed after 20 days of cells differentiation.

Biological Systems Regulated by miR-19 Validated Target Genes

As we were interested in investigating the biological processes and signaling targeted by miR-19a and miR-19b, we performed gene-targeting analyses using miRWalk2.0 database. Specifically, we looked at predicted as well as validated target genes of miR-19a, miR-19b-1, and miR-19b-2. We obtained lists of thousands of genes as predicted targets of all the three miRNAs (data not shown), a list of 631 validated target genes for miR-19a, a list of 787 validated target genes for miR-19b-1, and a list of 780

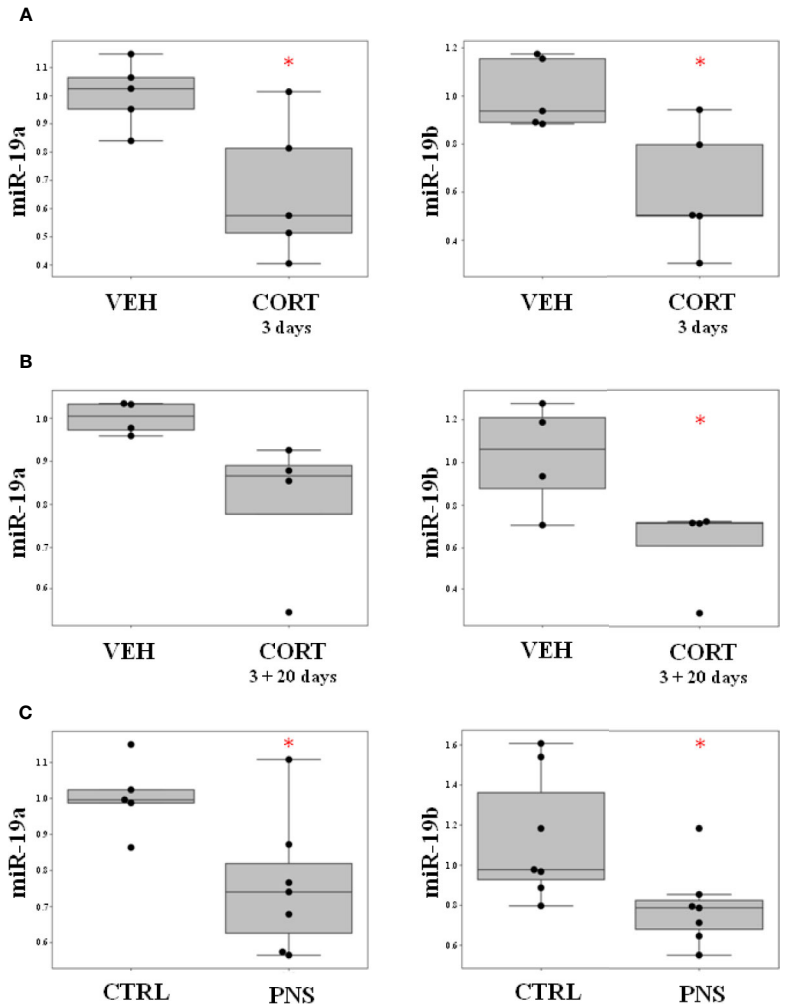

FIGURE 1 | (A) Effect of ELS on the modulation of miR-19a and miR-19b in HPC0A07/03C cells treated with cortisol during 3 days of proliferation as compared to cells treated with vehicle. **(B)** Effect of ELS on the modulation of miR-19a and miR-19b in HPC0A07/03C cells treated with cortisol during 3 days of proliferation and differentiated into mature neurons for 20 days as compared to cells that received vehicle. **(C)** Effect of PNS on the modulation of miR-19a and miR-19b in adult PNS rats (PND 62) as compared to control animals. Data are shown using boxplot, *p-value < 0.05.

validated target genes for miR-19b-2 (see **Supplementary Tables 1–3**). Thus, in order to restrict the datasets and to reduce the number of genes to focus on, we only considered the validated target genes.

We then performed a pathway analysis on each of these three lists of validated target genes by using IPA. We identified 229 statistically significant pathways potentially targeted by miR-19a, 220 potentially regulated by miR-19b-1, and 210 by miR-19b-2 that we listed in **Supplementary Tables 4–6**. In order to only select common pathways regulated by all the three miRNAs, we overlapped the three lists of pathways by using Venn diagram and we found 198 common significant pathways (**Figure 2A** and **Supplementary Table 7**), including, as the most representative ones, those involved in the *inflammatory and immune response* (i.e., TGFβ signaling, IL-6 signaling, NF-KB signaling, B cell receptor signaling, IL-2 signaling, T cell receptor signaling, Chemokine signaling), in *neurodevelopment* (i.e., Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency, NGF Signaling, P2Y Purigenic Receptor Signaling Pathway, Neuregulin Signaling, Synaptic Long Term potentiation, HIPPO signaling), as well as in the *intracellular signal transduction* (i.e., JAK/STAT signaling, ERK/MAPK signaling, ErbB signaling, PI3K/AKT signaling, AMPK signaling, RAC signaling, RhoA signaling). We also graphically represented all the significant common pathways in a pie chart detailing the relevant biological and functional processes (**Figure 2B**). Interestingly, among the most significant common pathways, we also found the Glucocorticoid Receptor Signaling (**Supplementary Table 7**), confirming the key role of miR-19 in stress response.

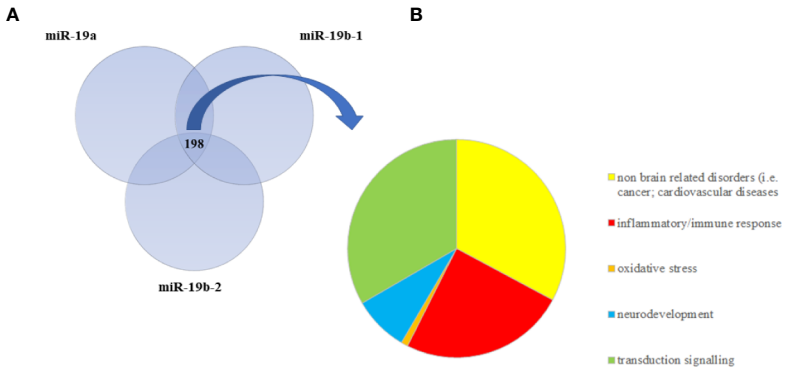

FIGURE 2 | (A) The Venn diagram represents the overlap between statistically significant pathways potentially regulated by validated target genes of miR-19a, miR-19b-1, and miR-19b-2. The intersection refers to 198 common significant pathways, which are mainly involved in inflammation, neurodevelopment, and intracellular signal transduction. **(B)** The pie chart summarizes the relevant biological functions of all 198 common significant pathways.

Bioinformatics Analyses of miR-19

Integrative analyses on open target platform (https://www.targetvalidation.org/) revealed a potential association of miR-19 target genes with MDD and SZ. Noteworthy, TNF60, PIK3R1, TP53, PINK1, NRCAM, IL4R, ADRB1, PTEN, and RAPGEF2 show an association with SZ at different level of evidence (genetic association, expression, and literature) and, interestingly, IL4R and ADRB1 have been found also associated with MDD. Moreover, TWAS analysis in SZ considering candidates gene from inflammatory and neurodevelopmental pathways suggest that genetic component of SZ [GWAS signal from (55)] is associated with the gene expression regulation of pivotal genes involved in these pathways (**Table 1**). Specifically, among the validated target genes, we selected TGFBR2, TNFα, IL4R, ADRB1, NRCAM, and PIK3R1 taking into account their involvement in pathways related to inflammatory and immune response (i.e., TGFBR2, TNFα, IL4R) and neurodevelopment (i.e., ADRB1, NRCAM, PIK3R1). We also selected PTEN and RAPGEF2 according to their well-established interaction with miR-19, as suggested by literature data (29, 30).

Noteworthy, the analysis of STRING interaction databases suggests an enrichment of interactions between the analysed genes and inflammatory/neurodevelopmental TWAS significant genes. In particular, the protein-protein interaction analysis shows an enrichment of interactions with TWAS genes involved in neurodevelopmental and inflammatory processes (p-value = 0.67E-04). This is further corroborated by the presence of a sub-cluster of such genes with at multiple levels of interactions evidences (see **Figure 3A**).

Effect of PNS on miR-19a and miR-19b Expression Levels in the Adult Offspring

In order to corroborate that stress early in life could have a long-term effect on the modulation of both miR-19a and miR-19b, we measured both miRNAs expression levels in the hippocampus of

TABLE 1 | Schizophrenia TWAS association of the analysed inflammatory and neurodevelopmental genes in blood and brain tissues.

Tissue	Gene	Z-score	p-value	Process
Brain Cerebellum	SLC25A12	4.27	1.93E-05	Neurodevelopment
Brain Cortex	SLC25A12	3.81	1.40E-04	Neurodevelopment
Brain Hippocampus	SLC25A12	3.42	6.23E-04	Neurodevelopment
Brain Cerebellar Hemisphere	TNIP1	−2.69	7.20E-03	Inflammation
Cerebellar Hemisphere	MAP3K9	2.61	9.18E-03	Inflammation
Whole Blood	DUTP6	2.50	1.25E-02	Neurodevelopment
Brain Hippocampus	STK4	2.41	1.59E-02	Inflammation
Brain Caudate basal ganglia	SNX27	2.37	1.79E-02	Neurodevelopment
Brain Caudate basal ganglia	DUTP6	2.29	2.18E-02	Neurodevelopment
Brain Nucleus accumbens basal_ganglia	FAS	2.18	2.95E-02	Inflammation
Brain Hippocampus	STK4	2.16	3.04E-02	Inflammation
Brain Frontal Cortex	RPA2	2.15	3.15E-02	Neurodevelopment
Whole Blood	RPA2	1.99	4.67E-02	Neurodevelopment
Brain Nucleus accumbens basal ganglia	DUTP6	1.98	4.75E-02	Neurodevelopment
Brain Cerebellum	LRP8	1.97	4.84E-02	Neurodevelopment

Only significant (p-value < 0.05) genes are reported in the table. Z-score represents the directionality of gene expression regulation (i.e., down- or upregulation).

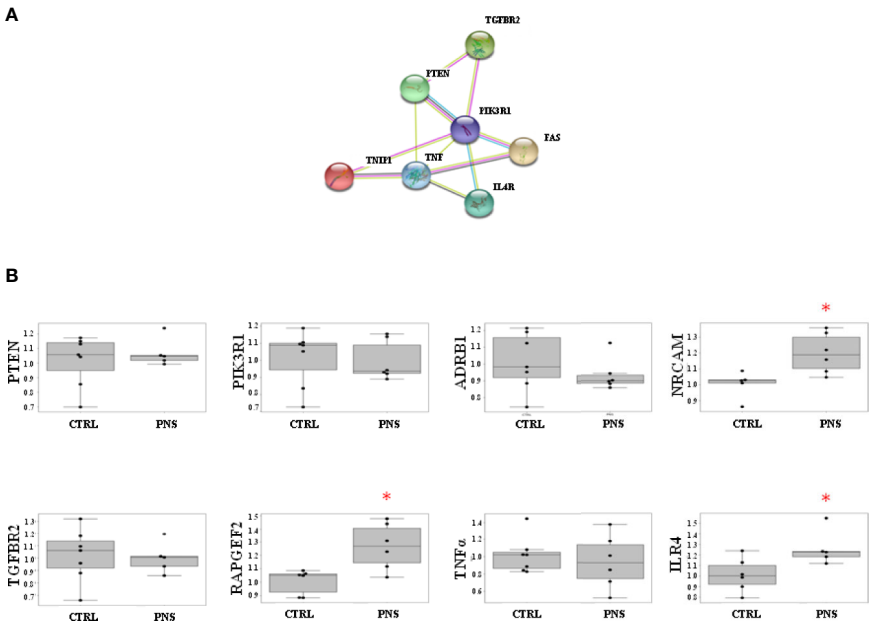

FIGURE 3 | (A) STRING protein-protein interactions between validated target genes of miR-19 selected for Real-Time PCR analyses. The protein-protein interaction analysis shows an enrichment of interactions with TWAS genes involved in neurodevelopmental and inflammatory processes (p-value = 0.67E-04), and is corroborated by the presence of a sub-cluster of such genes with at multiple levels of interactions evidences. **(B)** Effects of PNS on validated target genes of miR-19a and miR-19b in adult PNS rats (PND 62) as compared to control animals. PNS significantly upregulates the expression levels of NRCAM, IL4R, and RAPGEF2. Data are shown using boxplot; *p < 0.05.

adult male rats (PND 62) whose mothers were exposed to PNS during the last week of gestation as compared to controls.

Interestingly, we observed a statistically significant reduction in the expression levels of both miR-19a and miR-19b in the adult PNS offspring as compared to controls (FC = −1.35, p-value = 0.025 for miR-19a; FC = −1.43, p-value = 0.032 for miR-19b) (see **Figure 1C**).

Expression Levels of miR-19 Validated Target Genes in the Hippocampus of Adult PNS Rats

In order to investigate the modulation of miR-19 validated target genes, we have analyzed the mRNA levels of PTEN, PIK3R1, ADRB1, NRCAM, TGFBR2, RAPGEF2, TNFα, and IL4R in the hippocampus of adult PNS rats (PND 62) as compared to control animals. Specifically, we selected these validated target genes taking into account their involvement in pathways related to *inflammatory and immune response* (i.e., TGFBR2, TNFα, IL4R) and *neurodevelopment* (i.e., ADRB1, NRCAM, PIK3R1). We also selected PTEN and RAPGEF2 according to their well-established interaction with miR-19, as suggested by literature data (29, 30). Interestingly, among all the analyzed genes, we observed a significant increase of NRCAM, IL4R, and RAPGEF2 in the adult PNS rats as compared to controls (FC = 1.20, p-value = 0.027 for NRCAM; FC = 1.26, p-value = 0.046 for IL4R; FC = 1.23, p-value = 0.020 for RAPGEF2) (**Figure 3B**).

In addition, we performed correlation analyses between the modulation of miR-19a and miR-19b and the expression levels of these target genes in PNS rats and control animals, in order to identify potential associations between miR-19 and its targets. Overall, we observed inverse correlations between the mRNA levels of all the three genes and the levels of miR-19a and miR-19b, that however reached the significance only for RAPGEF2 and miR-19b (p-value = 0.019, r2 = 0.438). In addition, a trend of significance was observed between RAPGEF2 and miR-19a (p-value = 0.09, r2 = 0.318) and between ILR4 and miR-19a (p-value = 0.064, r2 = 0.409) (see **Figure 4**). However, these correlation analyses are not able to prove any causality, and further studies based on the use of functional constructs to block the effects of miR-19 on its targets will be helpful to prove the causative role of miR-19 in mediating the effects of ELS on biological processes and more importantly in shaping the future vulnerability of developing a specific phenotype of vulnerability.

DISCUSSION

In the present study, we observed that an exposure to ELS produces a short and long-term effect on miR-19 modulation, which in turn may affect normal trajectories of brain development and neuronal networks formation, increasing the vulnerability to develop psychiatric disorders later in life.

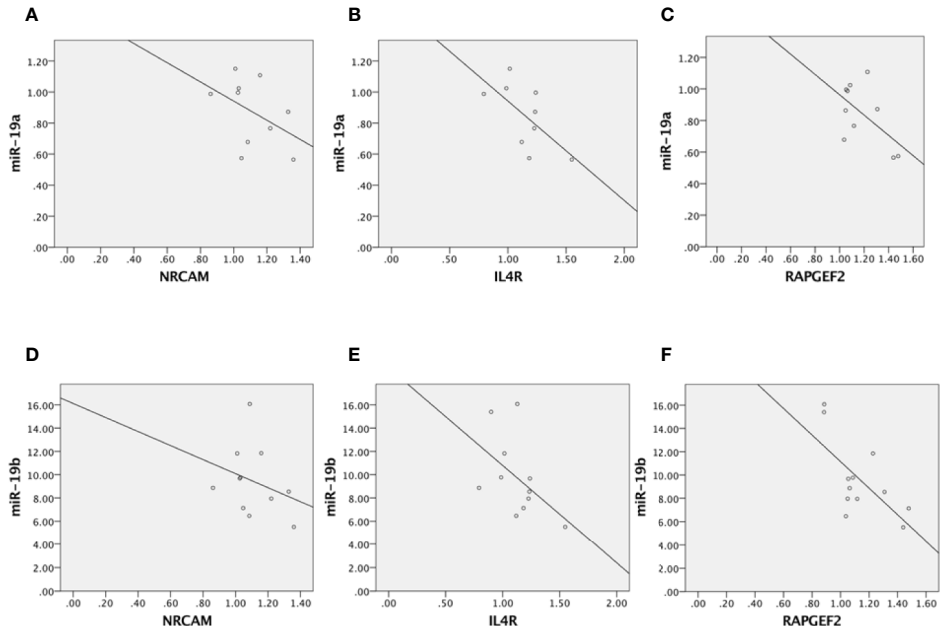

FIGURE 4 | Correlation analyses between miR-19a and NRCAM **(A)**, miR-19a and ILR4 **(B)**, miR-19a and RAPGEF2 **(C)**, miR-19b and NRCAM **(D)**, miR-19b and ILR4 **(E)**, and miR-19b and RAPGEF2 **(F)**.

According to a growing body of evidence, miR-19 expression is required for a proper neurodevelopment, as it controls the expansion of neural stem cells and radial glial cells, which are essential for normal cortical development and function (30). In addition, miR-19 has been suggested to be a key regulator of hippocampal neurogenesis (29, 38).

In human beings, the immature brain is highly plastic but particularly vulnerable to a range of environmental insults, including ELS, which can result in long-term cognitive and behavioral impairment (56). Indeed, a wide number of stressful environmental factors occurring during sensitive periods of life, such as maternal exposure to bereavement, natural disasters or terrorism as well as financial and relationship problems in pregnancy (57), or exposure to traumatic events in childhood (58), can compromise normal trajectories of brain development and neuronal networks formation, leading to abnormal programming of brain circuits (59).

Although the contribution of epigenetic mechanisms, including miRNAs, has been widely demonstrated in mediating the effects of ELS on brain functions (60, 61), to our knowledge, no studies have so far investigated the effects of ELS on miR-19 expression levels. Interestingly, in our previous paper (4), among the significantly modulated miRNAs identified through a miRNomics approach, we found miR-19a-3p as significantly downregulated in proliferating HPCs treated with cortisol and miR-19b-3p as significantly downregulated in the hippocampus of adult PNS exposed rats as compared to control animals. Based on these data, in this paper, we decided to follow up our results on miR-19 and to examine more in depth both the short- and long-lasting effects of ELS on the expression levels of this miRNA, the modulation of its variants and the involvement of possible biological signaling affected by miR-19 in association to ELS exposure.

Therefore, in this study, we first analysed the expression levels of miR-19a and miR-19b in our "*in vitro*" model of stress represented by HPC0A07/01C cells treated with high concentration of cortisol, a condition that we have previously found associated with a reduced neurogenesis. We observed that the levels of miR-19a and miR-19b were downregulated in proliferating cells treated with cortisol for 3 days as compared to vehicle. Moreover, the same effect was observed also in mature neurons, previously sensitized by cortisol, after 20 days of differentiation without any treatment. These data suggest that the expression levels of miR-19a and miR-19b begin to be modulated by cortisol during proliferation but persist overtime.

A similar result has been recently found by Provencal and collaborators, who reported an association between the exposure of HPCs to glucocorticoids at different stages (during proliferation and differentiation, but not after differentiation) and long-lasting changes in mRNA expression and DNA methylation profiles. Specifically, the long-lasting alterations in DNA methylation correlated with an enhanced responsivity to a second glucocorticoid challenge later in life (62), suggesting that early exposure to glucocorticoids may have a long-lasting impact on the development of the nervous system by priming the global expression levels of relevant genes. These findings suggest that prenatal exposure to glucocorticoids could not only alter

neurodevelopmental trajectories, but also change the set point of stress reactivity of adult tissues, increasing the risk for stress-related psychiatric disorders.

These data corroborate the so called "double hit" hypothesis, suggesting that ELS may act as a first negative "hit", predisposing individuals to be more vulnerable to subsequent negative stressful challenges, namely, the "second hit", later in life (63–65).

To further support our results in the *in vitro* model, we took advantage of a well-established animal model of PNS and we measured the expression levels of both miR-19a and miR-19b in the hippocampus of adult PNS male rats (PND 62) as compared to their controls, reporting a downregulation of both the miRNAs.

In line with our data, several studies have demonstrated that the exposure to prenatal or to early postnatal stress produces long-lasting alterations in the expression of molecules involved in biological systems important for brain plasticity. For instance, it is well known that rodents exposed to PNS show reduced expression levels of brain-derived neurotrophic factor (BDNF), a marker of neuronal plasticity (66). Accordingly, in a recent study performed by our group (67), a significant disruption in the novel object recognition test was found both in male and female adult rats exposed to PNS, although such impairment was more pronounced in females. The cognitive dysfunction observed during the behavioral test in adult PNS animals appeared to be the consequence of an abnormal activation of a pattern of genes, which was required for proper cognitive performances. Moreover, functional alterations originating from PNS exposure could represent the consequence of an inability to activate the proper transcriptional machinery required for the correct cognitive performance in relevant brain areas, such as the dorsal hippocampus. All these findings suggest that an exposure to stress early in life represents a priming event that interferes with the correct and physiological response during a cognitive task (67). Therefore, we suggest that the exposure to ELS not only influences the development of brain trajectories as well as the maturation of specific brain networks, but also affects cognitive impairments.

Although in the present study, PNS and control rats were not assessed for behavioural tests, previous studies also coming from our group have demonstrated that an exposure to PNS is associated with altered behavior in the offspring, both in adulthood (67, 68) as well as at peri-adolescence (66). Thus, the presence of lower levels of both miR-19a and miR-19b levels in the hippocampus of animals exposed to PNS could represent a biological signature of vulnerability that could shape the individual risk of developing altered behaviors and cognitive deficits later in life.

In order to investigate whether the biological processes regulated by miR-19a and miR-19b could be affected by ELS, we first performed gene-targeting analyses focusing only on validated target genes and subsequently we ran pathway analyses on these subsets of genes. We found that the most significant pathways modulated by miR-19a and miR-19b were involved in *neurodevelopment*, as we expected, but also in the *inflammatory/immune response* and in the *intracellular signal transduction*. In line with our results, a growing body of evidence has confirmed that ELS may alter the neuroinflammatory processes that interact with brain development, leading to a sensitized immune response and heightened neuroinflammation later in life, enhancing the risk for psychiatric disorders (69, 70). Accordingly, several clinical studies have demonstrated that ELS, both prenatally and in childhood, is associated with an increased inflammatory state, in terms of high C-reactive protein (CRP) and pro-inflammatory cytokines levels, later in life (71). This has been clearly described in the prospective cohort study performed by Baldwin and colleagues (72), who reported an association between childhood victimization, elevated levels of CRP at the age of 18 and the development of psychopathology later on. Similarly, a recent and interesting meta-analysis has clearly supported the association between the exposure to different types of childhood trauma and the presence of a pro-inflammatory state, represented by high CRP, Interleukin (IL)-6, and Tumor Necrosis Factor (TNF)-α levels (73).

To prove that the modulation of miR-19 could affect the long-term expression levels of pathways related to brain functions, stress response, and inflammation, we specifically analysed, in the hippocampus of adult rats exposed or not to PNS, the mRNA levels of a panel of genes targeted by miR-19 and involved in these signalling. Among all these validated target genes, we found increased expression levels of NRCAM, IL4R, and RAPGEF2 in PNS rats as compared to control animals at early adulthood, in line with a significant decrease in the mRNA levels of both miR-19 a and miR-19b observed in the same animals, as already described.

NRCAM, a member of the immunoglobulin superfamily, is involved in neural development including cell proliferation and differentiation, axon growth and guidance, as well as synapse formation. In the last years, genetic association studies have shown that alterations in NRCAM are associated with psychiatric disorders, such as SZ, autism, and drug addiction (74). Since NRCAM is involved in key brain functions and psychiatric disorders can be caused by the disruption of any of these processes, we can speculate that changes in the expression levels of this gene, possibly due to the modulation of miR-19, may introduce subtle but significant effects on brain development and wiring, which by themselves might be sufficient to increase the risk for psychopathologies.

Interleukin 4 receptor (IL4R) is a transmembrane protein that plays a critical role in binding IL4, a cytokine involved in several biological processes. Indeed IL4R not only regulates immune functions, such as IL4-mediated IgE production and Th2 inflammatory reactions (75), but it plays also a key role in pregnancy, fetal development, as well as in higher brain functions including memory and learning (76). Recently, IL4R has been also suggested to be directly involved in the promotion of neuronal survival and sprouting (77), thus preserving cognitive functions (77). Although IL4R is necessary for normal brain development (75), many aspects of neural IL4R expression, regulation, and signalling in both brain repair and disease remain to be examined.

By evaluating the association between fetal inflammation and neurodevelopmental delay at age 2, Clarke and collaborators have suggested that genetic alterations to the extracellular domain of IL4R may contribute to neurodevelopmental outcomes in pregnancies at high risk for preterm birth (78).

On these bases, we can hypothesize that changes in the expression levels of IL4R, following an exposure to ELS and possibly regulated by miR-19, may disrupt correct brain development and functions.

Finally, RAPGEF2 governs cell migration by modifying the activity of Rap proteins (79). As widely demonstrated, miR-19 drives cell migration by regulating RAPGEF2 expression levels in adult NPCs and several studies have shown an inverse correlation between the levels of miR-19 and of the target gene. Indeed, in physiological conditions, increased miR-19 levels suppress RAPGEF2 expression by binding to RAPGEF2 mRNA. Consistently with these findings, the migration efficiency of newborn neurons is increased when miR-19 is overexpressed and RAPGEF2 is depleted (Han and Gage, 2016). Based on this evidence, our results, showing that ELS decreases miR-19a and miR-19b expression levels already after 3 days of cell proliferation, and also after differentiation, might suggest that the presence of lower levels of miR-19 may not be able to suppress RAPGEF2 activity during the first steps of neural development, such as hippocampal neurogenesis. This could thus contribute in causing impairments in brain maturation and in rendering the system more vulnerable for the development of neurodevelopmental and stress-related disorders.

In conclusion, our data suggest that alterations in miR-19 levels as consequence of ELS exposure could cause alterations in pathways involved in neurodevelopment and in the immune system. Intervention strategies targeting miR-19 might be useful to prevent alterations in these pathways and to reduce the ELS-related effects. Of note, in our previous paper, we found a downregulation of miR-125b-1-3p in the same models used in this study and in particular in HPCs treated with high concentration of cortisol and in the hippocampus of PNS rats. Looking more in details at the possible biological systems modulated by target genes of these miRNAs, interestingly, we have observed that both miR-125b-1-3p and miR-19 are able to regulate pathways involved in the inflammatory and immune response and also in the neurodevelopment. Specifically, among the significant pathways regulated by both miR-125b-1-3p and miR-19, there are several pathways associated with inflammation and immune response, such as TGFβ signalling, CCR3 signalling in eosinophils, fMLP signalling in neutrophils, role of NFAT in regulation of the immune response. Only one common pathway, P2Y Purigenic Receptor Signalling Pathways, is more related to neurodevelopment. Altogether, these findings suggest that exposure to stress early in life can act on the modulation of several miRNAs, including miR-125b-1-3p and miR-19, which can play crucial roles in biological processes and functions involved in inflammation and immune response. These miRNAs can represent a possible epigenetic mechanism underlying the long-lasting effect of ELS that shape the individual vulnerability to develop stress-related psychiatric disorders later in life. However, further studies are needed to better dissect the contribution of these miRNAs and their common target pathways in association with ELS exposures.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Italian legislation on animal experimentation (D.L. 116/92).

AUTHOR CONTRIBUTIONS

MM, CrM, NM, VB, and NC contributed to the experiments in the *in vitro* and *in vivo* model. MM and NC managed the literature searches. MM, CaM, and NC performed the bioinformatics and statistical analyses. MM and NC contributed to the first draft of the manuscript, CP, MR, and AC contributed to the revision, and NC revised all the versions of the manuscript and approved the final one. All the authors have contributed to and have 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.2020.00389/full#supplementary-material

SUPPLEMENTARY TABLE 1 | List of 631 validated target genes of miR-19a.

SUPPLEMENTARY TABLE 2 | List of 787 validated target genes of miR-19b-1.

SUPPLEMENTARY TABLE 3 | List of 780 validated target genes of miR-19b-2.

SUPPLEMENTARY TABLE 4 | 229 statistically significant pathways regulated by miR-19a.

SUPPLEMENTARY TABLE 5 | 220 statistically significant pathways regulated by miR-19b-1.

SUPPLEMENTARY TABLE 6 | 210 statistically significant pathways regulated by miR-19b-2.

SUPPLEMENTARY TABLE 7 | List of 198 common significant pathways regulated by miR-19a, miR-19b-1 and miR-19b-2.

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The Role of Stress and Mineralocorticoid Receptor Haplotypes in the Development of Symptoms of Depression and Anxiety During Adolescence

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Adolescence is a critical developmental period characterized by heightened levels of depressive and anxiety symptoms. Experiencing chronic or environmental stress, for example, as a result of traumatic events or insensitive parenting, increases the risk for depression and anxiety. However, not all adolescents develop depressive or anxiety symptoms following environmental stressors, due to differences in stress resilience. One of the factors involved in stress resilience is enhanced functionality of the mineralocorticoid receptor (MR), one of the two brain receptors for the stress hormone cortisol. High levels of MR functionality result in relatively lower rates of depression, particularly in women that experienced stress. However, much less is known about MR functionality in relation to the development of adolescent depression and to other internalizing behavior problems such as anxiety. We therefore examined whether the effects of a functional MR haplotype (i.e., the MR CA haplotype) on the development of depressive and anxiety symptoms are sex-dependent, as well as interact with environmental stressors. In a community sample of adolescents (N = 343, 9 waves between age 13 and 24), environmental stressors were operationalized as parental psychological control and childhood trauma. Results showed a sex-dependent effect of MR CA haplotype on the development of depressive symptoms but not for anxiety symptoms. MR CA haplotypes were protective for girls but not for boys. This study sheds more light on the sex-dependent effects of MR functionality related to the development of depressive and anxiety symptoms during adolescence.

Keywords: depression, anxiety, mineralocorticoid receptor (MR), stress, adolescence, parenting, development

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INTRODUCTION

Adolescence is a vulnerable period for the development of internalizing behavior problems. While rates of depressive and anxiety symptoms are generally low in childhood, they increase to near-adult prevalence levels in adolescence (1, 2), with some studies showing a six-fold increase in rates of depression from age 15 to age 18 (1). Moreover, girls experience depressive and anxiety symptoms twice as often as boys (1, 3). High levels of stressors are a risk factor for the development of internalizing behavior problems (1, 4–6). In addition to the experience of stressful events, individual differences in stress resilience are consistently related to risk for psychiatric disorders (7). There is increasing evidence that the mineralocorticoid receptor (MR), one of the two receptors for the stress hormone cortisol, is important for stress resilience (8, 9). Functional MR receptor haplotypes (typical combinations of genetic variants with consequences for MR expression and activity), have been repeatedly found to affect the link between stressors and internalizing disorders (10, 11). Specifically, MR effects appear to be sex-dependent, with a stronger protective effect of increased constitutional MR activity for females compared to males (10, 11). However, much less is known about the relation of functional MR receptor haplotypes, environmental stressors, and sex with the development of depressive and anxiety symptoms during adolescence. We therefore investigated the possible sex-specific associations of the common and functional MR haplotype— both in presence and absence of environmental stressors—on depressive and anxiety symptoms during the crucial developmental period of adolescence.

In order to understand the consequences of MR haplotype and environmental stressors on the development of internalizing behavior problems such as depressive and anxiety symptoms, we focused on two common and salient environmental stressors during childhood or adolescence: childhood trauma in the form of physical abuse or neglect, and parental psychological control, which can be considered a form of emotional abuse. Childhood trauma and psychological control can have a long-lasting impact, which may become visible particularly during adolescence (6). When parents are psychologically controlling, they display disappointment and children feel pressured and guilty that they did not comply with the parent's requests, or anxious about losing the parent's approval (12). Also, childhood abuse is characterized by guilt and self-blame of the child (13). These processes may both result in loss of confidence and excessive inappropriate guilt, symptoms often present in internalizing disorders like depression (4, 14) and anxiety (3).

The MR is important for a well-functioning HPA axis. In response to stress, the HPA axis is rapidly activated and results in the release of cortisol (7). Cortisol binds to MRs in the brain and is essential for the activation and restoration of HPA axis activity in relation to stress (8, 15). Upon binding of cortisol, MRs translocate into the nucleus where they act as transcription factor by binding to responsive elements in promotor regions of target genes to increase gene expression (16, 17). Several clinical studies have pointed to a role of the MR in relation to the consequences of stress for depression. In rodents, decreased MR expression was associated with higher levels of depressive-like behavior (18) and rodents with elevated levels of MR functioning showed less anxious behaviors (19–22). In humans, MR studies with respect to internalizing behavior problems are scarce, and exclusively focus on depression. Several studies both in clinical and population-based adult samples have shown decreased MR expression in patients with Major Depressive Disorder (10, 23, 24). Also, lower rates of depression were found in human adults with higher MR expression (25).

MR is encoded by the NR3C2 gene, located on chromosome 4 (26). A sequencing study in a Dutch cohort identified two single-nucleotide polymorphisms (SNPs) in this gene, MR-2C/G (rs2070951) and MRI180V (rs5522), that affect the transactivational capacity in response to stress hormones (27). These SNPs constitute four possible haplotypes; CA, CG, GA, and GG, although the latter is very rare (28, 29). Mainly, the CA haplotype seems to be related to stress resilience. Having more CA haplotypes could therefore be protective against the negative consequences of environmental stressors and therefore the development of depressive and anxiety symptoms during adolescence. In this study, we will explicitly focus on the MR CA haplotype.

The MR CA haplotype has been shown to have sex-dependent effects on depression (10, 11). These sex differences may be explained by the fact that the female hormones estrogen and progesterone bind to the MR and affect MR functionality, although the exact underlying mechanism is unclear (30). Sex-specific effects of the MR may even be pronounced during adolescence when levels of female hormones increase and influence the maturing HPA axis (31).

In the current study, we therefore examined whether there is a sex-specific role of functional genetic variation in the MR (CA haplotype) for the development of depressive and anxiety symptoms during adolescence and young adulthood, and whether putative sex-specific MR effects depend on environmental stressors. We aimed to extend previous findings on sex-dependent MR effects on adult depression to the crucial developmental period of adolescence, and to extend the focus of MR on depression to anxiety as another important internalizing behavior problem. We hypothesized that the functional and common MR CA haplotype would have sex-dependent effects on the development of depressive and anxiety symptoms, and that the CA haplotype would be protective in girls. Moreover, we hypothesized that these protective effects of the MR CA haplotype in girls would be the most outspoken after exposure to environmental stressors.

METHODS

Participants

Participants were 343 Dutch adolescents (55.7% boys) with a mean age of 13 years (SD = .4) and all attending the first grade of secondary school at the first wave of data collection in 2005. Participants took part in the longitudinal population-based RADAR-Young study (Research on Adolescent Development

And Relationships), in which 522 adolescents originally participated. Adolescents were recruited in the center and west of Netherlands. The first six waves were annual until the age of 18 and the last 3 waves were biennial until the age of 24. All 343 adolescents that still participated at the age of 23 and for whom we collected valid and qualitatively good genetic data were included as participants of the current study. Adolescents from the genetic sample were not different from adolescents from the total sample on sex, age during the first wave, parental psychological control, childhood trauma, and depressive or anxiety symptoms (all *p*s < .05). For the participants in the genetic sample, there were few missing data, with 72% of the adolescents participating in all waves. Moreover, the pattern of missing values was estimated by Little's (32) Missing Completely at Random test. Although this test was significant ($\chi 2(N = 343, df = 709) = 844.39$, $p < 0.001$), the χ^2/df ratio of 1.19 indicated a good fit between the sample scores with and without imputation (33). Therefore, Full Information Maximum Likelihood was used to handle missing data in the growth curve analyses in M*plus* version 8.0. The study has been approved by the board of the local research institute and by the Medical Ethical Committees of the Utrecht Medical Centre and VU University Medical Centre, Netherlands, and written informed consent was obtained from both adolescents and parents.

Measures

MR CA Haplotypes

Genotyping of all participants was performed using Affymetrix 6.0 array's (34) on DNA from saliva. Two commonly investigated functional SNPs in the gene encoding the MR (rs2070951 and rs5522) were selected to derive MR haplotypes (15, 28). Rs5522 was genotyped and rs2070951 was imputed from the array data as previously reported (35). In short, all SNPs were strand aligned to the 1,000 genomes Phase 3 release, phased using SHAPEIT V2.970 and imputed to the 1,000 genomes reference with IMPUTE 2.3.1 following standard protocols. SNPs with R^2 values > .08 and average call rates > 0.99 were retained. Subsequently, SNPHAP was used to derive the haplotypes. This resulted in the following distribution of CA haplotypes: 41.40% adolescents with zero CA haplotypes, 46.36% with one CA haplotype, and 12.24% with two CA haplotypes, with more CA haplotypes indicating higher MR functioning, which is consistent with previous literature (11, 28, 29). Therefore, in the current study, we used the number of MR CA haplotypes as a continuous predictor. There were no significant differences in the haplotype distribution between men and women ($X^2 = 2.58$, $p = .276$).

Environmental Stressors

Parental Psychological Control

Psychological control was one of the environmental stressors. Psychological control involves attempts that intrude or manipulate the thinking processes, self-expression, and emotions of the child (36) and can therefore be considered as emotional abuse. It was measured by the Dutch version of the Psychological Control Scale (36), which consists of eight 5-point items on a 5-point Likert scale ranging from not at all applicable to completely applicable. An example item is "My mother acts like she knows what I'm thinking or feeling". Adolescents reported on psychological control by mother and father separately. All questions were completed from Wave 1 until Wave 7. At the last two waves, these questionnaires were not administered as many adolescents moved out of the parental home during the final waves. The internal consistency was high across waves, ranging from .75 to .89 for the reports about father and from .83 to .88 for the reports about mother. Power was too low to take parental psychological control into account as time-varying covariate. Therefore, for each adolescent, a mean score across the several waves and across fathers and mothers was calculated and standardized. Correlations between waves ranged from .37 to .76 for fathers, from .25 to .67 for mothers, and correlations between mothers and fathers on the same wave ranged from .56 to .72.

Childhood Trauma

To measure adolescents' traumatic events over the whole course of childhood and adolescence, the Dutch version of the Childhood Trauma Questionnaire-Short Form was assessed (37). This questionnaire was administered in the community sample at the ninth wave of data collection (around age 24). This retrospective questionnaire includes four 5-item subscales: physical abuse, emotional abuse, physical neglect, and emotional neglect, and one 4-item subscale: sexual abuse, reflecting the frequency of maltreatment on a 5-point Likert scale ranging from never true to very often true. A fifth item of the sexual abused scale "I believe I was molested" was not included in the Dutch version as there is no proper translation for the word "molested" with a sexual connotation (37). A total standardized continues score was calculated, and the internal consistency of the scale was adequate with a Cronbach's alpha of .83.

Depression

Depressive symptoms were measured by the Dutch adjusted version of the Reynolds Adolescent Depression Scale – 2nd edition (38). This scale consists of 23 items on a 4-point Likert scale ranging from 0 (almost never) to 3 (most of the time) on the subscales dysphoric mood (eight items), negative self-evaluation (eight items), and somatic complaints (seven items). An example item is "I feel bored". Items were averaged to compute a mean depression score. The internal consistency was high ranging from .93 to .95 on the different waves.

Anxiety

Anxiety symptoms were measured by the Screen for Child Anxiety Related Emotional Disorders (39). This scale consists of 38 items on a 3-point Likert scale ranging from 0 (almost never) to 2 (often), and consist of questions about somatic/panic, school phobia, social anxiety, generalized anxiety, and separation anxiety. An example item is "When I feel frightened, it is hard to breathe". Items were averaged to compute a mean anxiety score. The internal consistency of this scale was high ranging from .90 to .94 on the different waves.

Analyses

Growth curve analysis in M*plus* version 8.0 was used. To facilitate the interpretation of the effect of the predictor on the developmental trajectory of depressive and anxiety symptoms, a piecewise model was estimated to be able to examine the role of the predictors in different developmental periods. A separate slope was modeled for three developmental periods, resulting in three slopes: one for early adolescence (13–16 years), one for late adolescence (16–20 years), and one for young adulthood (20–24 years). Until age 18, an equidistant time difference of 1 was used for the annual measurements, and after age 18, an equidistant time difference of 2 was used for the biannual measurements. The intercept was the estimated amount of depressive or anxiety symptoms at age 13. The fit of this model was evaluated based on the following criteria: acceptable fit when CFI > .90, RMSEA and SRMR < .10, good fit when CFI > .95, RMSEA < .06 and SRMR < .08 (40).

We used the four latent factors (intercept and three slopes) of the piecewise model as dependent variables to examine the association between stressor, MR (0, 1, or 2 CA haplotypes), sex, and all two-way and three-way interactions with depressive symptoms and anxiety symptoms over the course of adolescence. Four different models were estimated, with either parental psychological control or childhood trauma as environmental stressor, and with either the latent factors of the growth curve of depressive symptoms or the latent factors of the growth curve of anxiety symptoms. This resulted in the following models: a) parental psychological control and depression, b) childhood trauma and depression, c) parental psychological control, and anxiety, d) childhood trauma and anxiety.

RESULTS

Descriptives and Model Fit

Depression

The piecewise growth model of depressive symptoms yielded a good fit (RMSEA = .062 [CI .043–.081], CFI = .967, SRMR = .032). In general, adolescents showed stable depressive symptoms between age 13 and 16 ($\beta_{slope13\text{-}16}$ = −.126, p = .089), after which their symptoms increased between age 16 and 20 ($\beta_{slope16\text{–}20}$ = .328, p < .001) and slowly increased between age 20 and 24 ($\beta_{slope20\text{–}24}$ = .144, p = .041), but with large individual differences in all parameters (all variances p's < .001). Also, the fit of the growth models with predictors was good, for both the model with parental psychological control (RMSEA = .048 [.033–.062], CFI = .970, SRMR = .023) and the model with childhood trauma (RMSEA = .040 [.025–.053], CFI = .973, SRMR = .029).

Anxiety

The piecewise growth model of anxiety symptoms yielded also a good fit (RMSEA = .043 [CI .019–.064], CFI = .982, SRMR = .058). In general, adolescents showed an initial decrease in anxiety symptoms between age 13 and 16 ($\beta_{slope13\text{–}16}$ = −.249, p = .006), after which their symptoms increased between age 16 and 20 ($\beta_{slope16\text{-}20}$ = .226, p = .005) and stayed stable between age 20 and 24 ($\beta_{slope20\text{–}24}$ = −.102, p = .154), but with large individual differences in all parameters (all variances p's < .001). Also, the fit of the growth models with predictors was good, for both the model with parental psychological control (RMSEA = .031 [.009–.047], CFI = .986, SRMR = .038) and the model with childhood trauma (RMSEA = .034 [.017–.048], CFI = .979, SRMR = .047).

MR, Stressor, and Internalizing Behavior Problems

Depression

We found no support for our hypothesis that the MR CA haplotype moderated the effects of environmental stressors on development of depressive symptoms. Specifically, there were no statistically significant three-way interactions between MR CA haplotype, stressors, and sex (all p's > .49, see **Table 1**), indicating no support for sex specific effects of MR in moderating the effect of environmental stressors. Similarly, in absence of sex, the interaction terms of MR CA haplotype with psychological control and MR CA haplotype with childhood trauma were both not significant for the intercept or slopes (all p's > .486).

However, in accordance to our hypothesis, we found significant negative interactions between MR CA haplotype and sex on the intercept ($\beta_{intercept}$ = −.20, p = .033 for psychological control, and $\beta_{intercept}$ = −.22, p = .032 for childhood trauma), but not the slopes (all p's > .340). This indicates that girls with more MR CA haplotypes reported lower levels of depressive symptoms across adolescence and young adulthood, while the opposite pattern was found for boys (see **Figure 1**).

Moreover, interaction between environmental stressors and sex were found. On the slope between age 16 and 20, there was a significant interaction effect between psychological control and sex ($\beta_{slope16\text{-}20}$ = −.29, p = .021), indicating that girls with higher levels of psychological control increased less in depressive symptoms as compared to girls with lower levels of psychological control, while depressive symptom development for boys was unaffected by parental psychological control. Second, the model with childhood trauma as stress factor showed a significant interaction effect between sex and childhood trauma on the intercept ($\beta_{intercept}$ = .31, p = .028) but not on the slopes (all p's > .394), indicating that girls with higher levels of childhood trauma reported higher levels of depressive symptoms across development.

Above these interaction effects, there were some main effects. Most consistently, sex was found to predict levels of depressive symptoms, with girls showing more depressive symptoms at age 13 ($\beta_{intercept}$ = .36, p < .001 for the parental psychological control model, $\beta_{intercept}$ = .41, p < .001 for the childhood trauma model). In the childhood trauma model, an effect of sex on the second slope ($\beta_{slope16\text{-}20}$ = −.19, p = .048) indicated that after an initial higher level of depressive symptoms for girls, between the age of 16 and 20 depressive symptoms in girls increased to a lesser

TABLE 1 | Standardized regression coefficients and standard errors of MR, sex, parental psychological control (PCS), and childhood trauma (CTQ) as predictors of the development of depressive symptoms.

	Intercept		Slope 13–16		Slope 16–20		Slope 20–24	
	β	SE	β	SE	β	SE	B	SE
Parental Psychological control								
MR	.12	.07	−.13	.08	.04	.09	.00	.10
Sex	.36***	.09	.10	.11	−.17t	.09	.06	.10
PCS	.24*	.10	.18	.12	.02	.12	−.17	.14
MR*sex	−.20*	.09	.11	.12	−.10	.11	−.02	.11
MR*PCS	.02	.10	−.06	.13	−.04	.12	.09	.13
PCS*sex	.19	.13	.12	.15	−.29*	.12	−.02	.14
MR*sex*PCS	.08***	.11	−.02t	.13	.07*	.12	−.05	.13
R^2	***.31******		***.10t***		***.12****		***.03 n.s.***	
Childhood trauma								
MR	.13t	.07	−.10	.09	.04	.09	.00	.09
Sex	.41***	.09	.14	.11	−.19*	.10	.04	.09
CT	.02	.09	.31**	.11	.10	.12	−.11	.11
MR*sex	−.22*	.10	.08	.12	−.08	.11	−.02	.11
MR*CT	.18t	.10	−.02	.11	−.10	.18	.16	.15
CT*sex	.31*	.14	−.12	.22	.00	.14	.12	.14
MR*sex*CT	−.11**	.13	.04*	.19	−.14t	.18	−.17	.17
R^2	***.22****		***.11****		***.09t***		***.01 n.s.***	

*t < .10; *< .05; **< .01; ***< .001; ns, not significant. Sex was coded as boys = 0, girls = 1.*

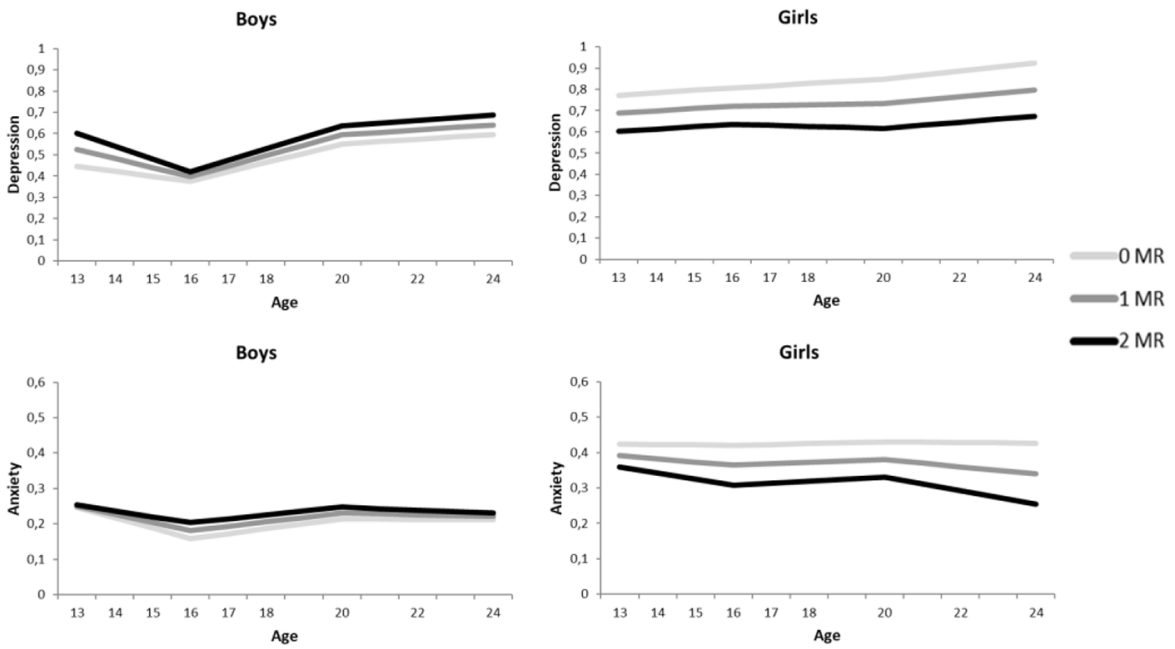

FIGURE 1 | Growth curve models for the depressive symptoms (above) and anxiety symptoms (below), separately for boys (left) and girls (right). The darker the lines the more MR CA haplotypes.

extent compared to boys (potentially due to higher initial or overall levels of depressive symptoms in girls). This effect did not reach significance in the model for parental psychological control ($\beta_{slope16\text{-}20}$ = −.17, p = .077), although effect sizes were comparable to the model with childhood trauma. Also, while psychological control was significantly related to more depressive symptoms across development ($\beta_{intercept}$ = .24, p = .019, slopes all p's > .135), childhood trauma did predict a steeper slope in depressive symptoms from age 13 to 16 ($\beta_{slope13\text{-}16}$ = .31, p = .005).

TABLE 2 | Standardized regression coefficients and standard errors of MR, sex, parental psychological control (PCS), and childhood trauma (CTQ) as predictors of the development of anxiety symptoms.

	Intercept		Slope 13–16		Slope 16–20		Slope 20–24	
	β	SE	β	SE	β	SE	β	SE
Parental Psychological control								
MR	.01	.07	.04	.08	−.02	.08	−.03	.07
Sex	.35***	.09	.20t	.11	−.12	.12	.02	.11
PCS	.21t	.11	−.02	.11	−.07	.11	−.19	.13
MR*sex	−.07	.11	−.11	.14	.03	.13	−.10	.12
MR*PCS	−.09	.11	.07	.12	.00	.11	.13	.11
PCS*sex	.19	.15	.21	.14	−.22	.16	−.03	.15
MR*sex*PCS	.07***	.13	−.12	.16	.10	.15	−.05	.13
R^2	***.25******		***.05 n.s.***		***.06 n.s.***		***.04 n.s.***	
Childhood trauma								
MR	.01	.07	.06	.08	−.02	.08	−.03	.07
Sex	.40***	.09	.21t	.11	−.14	.11	−.01	.11
CTQ	.04	.09	.07	.10	.06	.13	−.17	.11
MR*sex	−.09	.11	−.11	.13	.04	.13	−.08	.11
MR*CTQ	−.05	.10	.10	.10	−.07	.14	.16	.10
CTQ*sex	.36*	.14	−.06	.16	−.07	.23	.06	.16
MR*sex*CTQ	−.07**	.14	−.06	.17	.06	.24	−.19	.17
R^2	***.23****		***.03 n.s.***		***.02 n.s.***		***.04 n.s.***	

*t < .10; *< .05; **< .01; ***< .001; ns, not significant. Sex was coded as boys = 0, girls = 1.*

Anxiety

In accordance with the models of depression, the anxiety models showed no indication of an interaction between MR CA haplotype and environmental stressors, or three-way interactions between MR CA haplotype, sex and environmental stressors on the development of anxiety symptoms (all *p*'s > .233, see **Table 2**). In contrast to depression, no interaction between MR CA haplotype and sex was found on the intercept or the three slopes (all *p*'s > .395). Although **Figure 1** suggests an interaction between MR CA haplotype and sex, the effects are too small to reach significance.

Comparable to the models for depression, there was an interaction effect between childhood trauma and sex ($\beta_{intercept}$ = .36, p = .010), but not between parental psychological control and sex ($\beta_{intercept}$ = .19, p = .197), on the intercept of anxiety symptoms. These findings indicate that girls with higher levels of childhood traumas reported higher levels of anxiety symptoms across development.

There were a couple of main effects, for which all effect sizes were comparable to the models for depression. Both anxiety models showed a main effect of sex on the intercept ($\beta_{intercept}$ = .35, p < .001 for parental psychological control, and $\beta_{intercept}$ = .40, p < .001 for childhood trauma), indicating that girls reported higher levels of anxiety as compared to boys across development. Moreover, parental psychological control showed a marginally significant effect on the intercept of anxiety symptoms ($\beta_{intercept}$ = .21, p = .056), with an effect size comparable to depression, suggesting that adolescents that experience higher levels of parental psychological control also reported higher levels of anxiety. In contrast to the depression model, there was no indication of a main effect of childhood trauma on the intercept or the three slopes of anxiety (all *p*'s > .102).

DISCUSSION

In this longitudinal study of a community sample assessed during adolescence and young adulthood, we found that common and functional MR haplotypes had sex-dependent protective effects on depressive symptoms but not on anxiety symptoms. Specifically, we found that girls with the MR CA haplotype consistently had lower depressive symptoms compared to non-CA haplotype carriers. The MR CA haplotype was a *protective* factor for mean levels of depressive symptoms of girls across adolescence and young adulthood, but a *risk* factor for boys, independent of the level of environmental stressors. Findings support earlier evidence for sex-dependent effects of the functional and common MR CA haplotype on depression. With regard to environmental stressors, we found no support for a moderating role of MR CA haplotype in the effects of environmental stressors on depressive and anxiety symptoms during adolescence, and no sex-specific effects of environmental stressors were found in relation to MR CA haplotype. This study adds to previous findings by shedding more light on the sex-dependent effects of the MR for the development of internalizing behavior problems in relation to stressors during the turbulent period of adolescence.

There were no significant interaction effects between stressors and MR CA haplotype, suggesting that MR CA haplotype is equally protective in low and high stressful environments. However, a study in the same sample showed a positive effect of MR CA haplotype on prosocial behavior, empathic concern and perspective taking for adolescents who experienced high levels of stressors, and negative effects for adolescents who reported low levels of stressors (41). Such a protective effect of MR CA haplotype under circumstances of high stress is in line with the study of Vinkers et al. (11) that found stronger MR CA

haplotype effects for higher levels of childhood trauma. This suggests that the MR receptors will mainly be occupied and provide feedback to the HPA axis when stress hormone levels are high and therefore are mainly relevant under circumstances of high stress (8, 15). As such, MRs would be mainly protective in adolescents with high levels of stressors by affecting the appraisal of and response to a stressful situation (9).

One possible explanation for why our study did not find a significant interaction between MR CA haplotype and stressors could be due to the way stressors were measured. First, both questionnaires assessing parental psychological control and childhood traumas assessed the amount of stressors, and not the amount of stress it created for the adolescent. When someone experiences more stressful events, or a longer period of stress, their stressful event load accumulates during lifetime (42), while at the same time the effects of stressful experiences decrease over time (42–44). Therefore, a certain stressor during childhood or adolescent might result in different levels of stress at different points in development. Moreover, by taking one score for the whole period of adolescence and adulthood we lost information about the timing of the stressors. Second, the level of stressors in our community sample was relatively low, which made it less likely to detect effects. Third, both questionnaires mainly focused on parental maltreatment, while other forms of stressful events that might have contributed to adolescents' stress load and the development of internalizing behavior problems were not taken into account, like peer-victimization (45). Future studies could provide more insight in this process by measuring a broader range of stressful events, asking participants to report the level of stress associated with childhood trauma or parental psychological control, and asking the exact timing and duration of these stressful experiences.

The protective role of MR CA haplotype in girls is in line with earlier studies that found sex-specific effects of MR CA haplotype related to the development of depression in a population sample (10, 11). In line with our results, they found that females with MR CA haplotype were protected against the development of depression, and that males with MR CA haplotype were at increased risk for depression. This sex-specific MR effect may be understood from the fact that the female hormones progesterone and estrogen influence MR functioning (30). This makes females differentially susceptible to the consequences of stress and therefore for the development of depression and other internalizing behavior problems. For example, females with the MR CA haplotypes were found to be less sensitive to their female hormonal status with respect to emotion recognition (46). In line with this finding, females with MR CA haplotypes were protected against the negative effects of oral contraceptives on recognition of sad and fearful faces and worse emotional memory (47). These findings suggest that sex interacts with MR in such way that MR CA haplotype is more protective in women, but that the sex-specific MR effects might depend on female hormone levels. To gain better understanding of the exact sex-specific MR effects on depressive and anxiety symptoms, future studies should take into account the actual hormone levels or focus on the underlying biological mechanism for the sex-specific MR effects.

For girls, MR CA haplotype had a constant significant protective effect on the level of depressive symptoms across the whole period of adolescence and young adulthood, rather than being related to change in depressive symptoms across time. It might be that MR CA haplotype already has an effect on depressive symptom development before the start of adolescence. As MRs are important for an optimal stress response (8, 15), the functional and common MR CA haplotype may already affect stress resilience from birth on, and consequently, the development of the earliest symptoms of depression or other internalizing behavior problems. For example, coping style and temperament are found to interact with environmental stressors and moderate the risk of depression (42). It might be that stress resilience due to a MR CA haplotype is related to a more adaptive coping style or certain temperamental inclinations in young children, and therefore results in lower rates of depression. Childhood studies about the role of MR in internalizing behavior problems could provide more insight in the putatively protective role of MR CA haplotype in the earliest symptoms of depression in relation to hormonal status in males and females.

Although sex-specific MR effects were found for development of depressive symptoms, there was no sex-specific MR effect for the development of anxiety symptoms. This in accordance with studies in which HPA axis functioning and cortisol levels have been consistently related to depression (48–51), but less so to anxiety or only to some forms of anxiety (52). As MR is relevant for the activation and restoration of the HPA axis by binding to cortisol (8, 15), findings for MRs may be comparable to the results of HPA axis functioning and cortisol for anxiety. In line with the findings of Vreeburg et al. (52), anxiety problems are diverse (3), and MR might only relate to the development of certain anxiety symptoms. Moreover, meta-analyses on parenting showed that in general the effects of stressful parenting are smaller for anxiety compared to depression (53, 54), which suggests that the role of MR in the relation between parenting stressors and the development of anxiety symptoms is also smaller. Future research that examines a wide range of internalizing behaviors could shed more light on the extent to which MR CA haplotypes play a role in the development of internalizing behavior problems.

An important strength of our study is the longitudinal investigation of consequences of MR functioning. Whereas most previous research has been cross-sectional and focused on adult populations, we were able to investigate the development across adolescence into adulthood. Moreover, this study was the first that examined MR functioning in relation to other internalizing behavior problems than depression. Despite these strengths, this study also had several limitations. First, we used self-report questionnaires for all constructs. Adolescents' subjective perceptions of their trauma, the parenting they receive, and their internalizing symptoms are considered the most important information, but using other-reports might have strengthened our findings by providing triangulation. Another disadvantage is that the studied genetic variation was limited to one MR haplotype instead of more extensive genetic variation or

HPA axis functionality. However, as earlier studies showed the importance of MRs for stress resilience (8, 9), with a pronounced role for the MR CA haplotype (29), this study on sex-dependent effects of this common and functional MR haplotype was important to gain more insight in why some adolescent are more stress-resilient than others in the development of depressive and anxiety symptoms. Finally, although the sample is quite large for a longitudinal study that covers the developmental period from early adolescence to young adulthood across nine measurement waves, the number of participants might be considered relatively low for analyses including multiple interaction terms. Consequently, we should be cautious with interpretation of our findings given potential low power and relatively few significant associations.

This study showed that MR haplotypes are relevant for the development of depressive symptoms in a community sample, with a protective effect of the MR CA haplotype in girls. No evidence was found for a role of MR in anxiety symptom development, but research on a wider range of internalizing behaviors is needed to clarify the role of MR in the development of internalizing behavior problems. Also, the interaction between MR CA haplotype and environmental stressors warrants further investigation which should take into account biological responses to the timing of the stressor and the amount of stress caused by the stressors. Moreover, in future studies, a more extensive focus on the childhood and early adolescent period, and more details on the role of sex and sex-specific hormones, are needed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committees of the Utrecht Medical Centre and VU University Medical Centre, Netherlands, and Ethical Committee of Faculty of Social Science of the Utrecht University, Netherlands. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HE: Formal analysis, writing—original draft, visualization. SN: Formal analysis, writing—review and editing. RS: Formal analysis, writing—review and editing. MB: Conceptualization, writing—review and editing. PL: Conceptualization, writing—review and editing. WM: Conceptualization, writing—review and editing. SB: Conceptualization, writing—review and editing supervision. CV: Conceptualization, writing—original draft, supervision.

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Childhood Physical Neglect Is Associated With Exaggerated Systemic and Intracellular Inflammatory Responses to Repeated Psychosocial Stress in Adulthood

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Experiences of child maltreatment are associated with a host of adverse mental and physical health outcomes in adulthood. Altered reactivity to psychosocial stress exposure may partially explain known associations between early experiences of maltreatment and later life health. The present study focuses on examining whether experiences of child maltreatment are associated with physiological reactions to initial and repeated psychosocial stress in adulthood. To this end, 44 healthy adults (52% male, aged 18–65) completed the Childhood Trauma Questionnaire to provide information about exposure to child maltreatment and completed the Trier Social Stress Test (TSST) on 2 consecutive days. Peripheral blood was collected prior to as well as 30 and 120 min following the TSST on each day. Plasma Interleukin-6 (IL-6) and gene expression of IL-6, IL-1β, nuclear factor-kB (NF-kB), and inhibitor of kB (IkB) were measured from each blood sample. Total CTQ scores were unrelated to plasma IL-6 and gene expression (*p*s > .10) but a history of childhood physical neglect was associated with increased interleukin-1β (β =.35; *p* =.02; R^2 =.19) and nuclear factor-kB (β =.30; p =.046; R^2 =.13) expression following initial stress. Following repeated exposure to the TSST, childhood physical neglect was associated with increased plasma IL-6 reactivity (β =.34; p =.02; R^2 =.16) and increased expression of nuclear factor-kB (β =.31; p =.04; R^2 =.08). Finally, childhood

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physical neglect was associated with decreased habituation following repeated exposure to the TSST. Other CTQ subscales were not related to plasma IL-6 and gene expression when considered individually. Results from this study are suggestive of a unique effect of childhood physical neglect on the physiological stress response following initial and repeated exposure to a common psychosocial stressor. This provides important directions for future research because the effect of childhood physical neglect on long-term neglect are not well understood and in need of further investigation.

Keywords: childhood maltreatment, abuse, neglect, inflammation, gene expression, stress reactivity, Trier Social Stress Test, repeated stress

INTRODUCTION

Child maltreatment experiences, including both exposure to abuse and neglect, continue to be very common. In the United States, more than one third of youth are investigated for possible child maltreatment before they reach age 18 (1). Existing research has clearly linked exposure to adverse childhood experiences, such as maltreatment, to poorer health outcomes during adulthood, including, e.g., increased risk of chronic health problems, such as obesity, cardiovascular disease, and cancer (2–5), and to all-cause mortality (6).

More needs to be understood, however, about the physiological pathways linking experiences of child maltreatment to worse health outcomes decades later. To this end, previous studies have primarily focused on understanding changes to hypothalamic-pituitary-adrenal (HPA) axis functioning, which plays an important role in controlling inflammatory responses, as well as on inflammatory outcomes directly (7). Adults with a history of child maltreatment experiences have been shown to exhibit higher levels of low-grade systemic inflammation (8–10). Given that the influence of greater systemic inflammation on numerous chronic diseases of aging has been well established, e.g., for risk of obesity, diabetes, and cardiovascular disease (11–13), altered inflammatory functioning is a likely culprit for connecting early experiences of maltreatment to subsequent poorer health. These observations are in line with the general hypothesis that experiences of child maltreatment program peripheral white blood cells, in particular monocytes, toward more pro-inflammatory phenotypes and reduced sensitivity to the immunosuppressive effects of glucocorticoids, such as cortisol (14).

Although associations between child maltreatment and low-grade inflammation have been shown repeatedly in the context of observational studies, fewer data are available on acute stress-induced changes in inflammatory cytokines. For example, healthy adults with a self-reported history of child maltreatment showed greater plasma IL-6 responses to an acute psychosocial stressor in the lab compared to those who did not report a history of child maltreatment (15). Even less is known about possible acute stress effects at the intracellular level that might facilitate a pro-inflammatory stress response. Exposure to acute stress has measurable effects at the intracellular level, however, including increases in DNA binding activity of nuclear factor (NF)-kB (16–18) and the subsequent transcription of inflammatory genes (19). Although not focused specifically on child maltreatment, existing studies have linked acute psychosocial stress exposure to greater pro- and anti-inflammatory gene expression (14, 18, 20, 21).

The present study aims to expand on previous research in a number of important ways. First of all, we set out to examine the association between self-reported child maltreatment history specifically and both plasma interleukin-6 (IL-6) levels and inflammatory gene expression responses to an established acute psychosocial laboratory stressor, the Trier Social Stress Test (TSST) (22). Based on existing research, we hypothesize that individuals with a self-reported history of child maltreatment will show evidence of greater plasma IL-6 and inflammatory gene expression responses following acute stress exposure compared to those without a history of child maltreatment.

Second, because acute stressors are not isolated incidents in real life, and perhaps even less so among individuals with a history of child maltreatment, we examine the effects of repeated exposure to this stressor on plasma inflammation and gene expression responses. Marked habituation to repeated stress exposure has previously been shown for four select pro- and anti-inflammatory gene products, i.e. IL-1β, IL-6, NF-kB and inhibitor of kappaB (IkB) following repeated stress exposure in healthy participants (21). However, failure to habituate to repeated stress exposure may contribute to a more pro-inflammatory state, thereby increasing disease susceptibility later in life. Thus, we hypothesize that individuals who report a history of child maltreatment will be less likely to habituate to repeated stress exposure.

Third, most research focusing on the physiological consequences of child maltreatment focuses only on child maltreatment experiences broadly, on either only male or female participants, or only on individuals who have experienced particular types of child maltreatment, most commonly sexual or physical abuse (e.g., (5, 23–27). Consequently, although neglect is the most common form of child maltreatment, making up approximately 70% of all child maltreatment cases in the United States, its effects on long-term health outcomes are completely understudied. The present study not only includes both male and female participants, but also, in additional exploratory analyses, examines the effect of maltreatment type on physiological responses to repeated acute stress exposure. Given the absence of research on the

physiological consequences of childhood neglect, specific hypotheses regarding maltreatment type are difficult; however, given the widely reported adverse effects of sexual abuse history, we hypothesize that sexual abuse in particular may be associated with increased inflammatory responses to acute stress.

MATERIALS AND METHODS

Participants

Participants were a total of n = 44 healthy adults, ages 18–65 years (M = 37.96 ± 17.43 years; 52% male; mean body mass index (BMI) = 24.94 kg/m^2 ± 3.01; mean body fat = 25.33% ± 6.54), recruited from the Greater Boston area and the Brandeis University campus. All participants underwent a brief medical and psychological screening before testing and met the following inclusion criteria: (a) BMI between 18 and 35 kg/m^2; (b) for females, luteal phase of menstrual cycle at time of participation, because cortisol stress responses impact inflammation and vary throughout the menstrual cycle (28); (c) absence of psychiatric, endocrine, cardiovascular, inflammatory, or other chronic diseases; (d) no use of psychoactive drugs, anti-inflammatory drugs, beta-blockers, gonadal steroids (i.e., hormonal contraceptives), glucocorticoid medications; (e) non-smoker, and (f) no previous experience with the stress protocol. See **Table 1** for detailed participant characteristics.

Procedure

Eligible participants were scheduled for laboratory sessions on two consecutive days between 13:30–18:30 h to control for circadian variation of stress hormones. Participants were instructed to refrain from eating or drinking anything but water for 1 h before the laboratory sessions. Written informed consent was obtained prior to participation. Each laboratory session lasted 3 h and included exposure to the TSST (22). Blood was drawn from an antecubital vein using a peripheral venous catheter (BD Nexiva IV catheter, Becton–Dickinson, Franklin Lakes, NJ) and collected in EDTA Vacutainer tubes for measurement of plasma IL-6 (Becton–Dickinson), and Tempus Blood RNA tubes (Life Technologies, Carlsbad, CA) for RNA. Placement of the catheter was followed by a 30-min resting period to ensure recovery from any potential stress response. Blood was drawn at baseline (pre-TSST), as well as 30 and 120 min following the TSST on both study days. The Brandeis University Institutional Review Board approved all procedures.

TABLE 1 | Participant characteristics.

	N (%)	Mean ± SD
Age (years)		37.96 ± 17.43
Sex (male)	23 (52)	
Race/ethnicity		
White	22 (50)	
Asian	8 (18)	
Black	5 (11)	
Other	9 (20)	
Body mass index		24.94 ± 3.01
Body fat percentage		25.33 ± 6.54
Depressive symptoms (CESD)		13.30 ± 10.59

Stress Induction Paradigm

Acute psychosocial stress was induced using the TSST (22), a widely used standardized laboratory stress paradigm. Following a well-established and commonly used paradigm, the TSST used in the present study consisted of a 3-min preparation period, a 5-min public speech in the form of a job interview, and a 5-min mental arithmetic task in front of an audience of two judges wearing lab coats and maintaining neutral evaluative facial expressions as previously reported (21, 29). Full details regarding the TSST are available elsewhere (21, 29). Briefly, all participants were given the same prompts and completed the same tasks in the same order. During the 3-min preparation period, participants were left alone in a room with a pencil and paper to prepare for their speech. If participants ended their speech before the 5 min were up, the judges asked them to "please continue" but did not interact with participants otherwise. At the end of the 5-min speech period, participants were thanked for their speech and informed that their time was up. For the arithmetic task, participants were asked to subtract the number 13 beginning at 1,022 as quickly and accurately as possible. Participants were asked to start over following any arithmetic mistake they made.

Self-Report Measures

Child Maltreatment

Exposure to child maltreatment was assessed using the Childhood Trauma Questionnaire (CTQ) (30). The CTQ includes 25 items to assess five types of child maltreatment, specifically physical abuse (e.g., "I got hit so hard by someone in my family that I had to see a doctor or go to the hospital"), sexual abuse (e.g., "Someone tried to touch me in a sexual way, or tried to make me touch them"), emotional abuse (e.g., "People in my family called me things like "stupid," "lazy," or "ugly"), physical neglect (e.g., "I had to wear dirty clothes"), and emotional neglect (e.g., "My parents were too drunk or high to take care of the family"). Responses were given on 1 ("never true") to 5 ("very often true"). Internal reliability of the CTQ for the present sample was good (Cronbach's $\alpha = 0.81$).

Scores were computed for each subscale by summing the respective items, with possible ranges from 5 to 25. Additionally, the CTQ yields a sum score for overall adversity, with a possible range of 25–125. CTQ scores were used continuously for main correlation and regression analyses. Dichotomous CTQ scores comparing individuals scoring above and below previously established cut-offs (31) were also used. Cut-offs for individual subscales were as follows: physical abuse, physical neglect, and sexual abuse: 8; emotional neglect: 15; emotional abuse: 10. Due to the relatively low level of adversity in this sample, the cut-offs produced uneven group distributions for several of the subscales. Consequently, a dichotomous total CTQ score was created. Participants were considered to have experienced "any" abuse if they scored above the cut-off for any one (or more) of the subscales and considered to have experienced "no" abuse if they did not score above the cut-off for any subscales. See **Table 2** for

TABLE 2 | Mean, range, and cut-off distributions for the Childhood Trauma Questionnaire.

	Mean (SD)	Observed Range	Cut-off Score[1]	Below cut-off (male/female)	Above cut-off (male/female)	No maltreatment (male/female)	Any maltreatment (male/female)
Physical Abuse	7.02 (4.2)	5 to 25	8	31 (17/14)	13 (6/7)	20 (12/8)	24 (11/13)
Emotional Abuse	9.57 (4.4)	5 to 22	10	25 (15/10)	19 (8/11)	11 (6/5)	33 (17/16)
Sexual Abuse	6.37 (3.6)	5 to 25	8	37 (21/16)	7 (2/5)	35 (21/14)	9 (2/7)
Physical Neglect	6.76 (3.1)	5 to 19	8	36 (20/16)	8 (3/5)	29 (17/12)	15 (6/9)
Emotional Neglect	11.00 (4.5)	5 to 24	15	34 (20/14)	10 (3/7)	5 (3/2)	39(20/19)
CTQ total	48.97 (11.1)	37 to 73	48	25 (15/10)	19 (8/11)	11 (6/5) [2]	33 (17/16) [2]

[1]*Cut-off scores based on established range by Walker et al. (31).*

[2]*No maltreatment vs. any maltreatment based on whether participants reported maltreatment above the cut-off for no vs. any of the subscales, respectively.*

the distribution of participants across groups based on these criteria.

Depressive Symptomatology

Depressive symptoms were assessed using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D) (32) and adjusted for in all analyses. The CES-D has demonstrated reliability and validity (32). Items are answered on a 4-point Likert scale and responses averaged to produce an overall score. Higher scores reflect greater depressive symptoms, and a score of 16 is widely accepted as a clinical cut-off. In the present sample, 64% of participants scored below the clinical cut-off (M = 13.3; SD = 10.6). Internal reliability in the present sample was very good at Cronbach's $\alpha = 0.93$).

Inflammatory Stress Responses

Systemic Inflammation

Plasma IL-6 was assessed at baseline (pre-TSST), as well as 30- and 120-min post-TSST on both study days. Peripheral blood samples were centrifuged immediately following collection and plasma was aliquoted and stored at −80°C. IL-6 concentrations were determined using commercially available high-sensitivity ELISA kits (Quantikine HS; R&D Systems, Minneapolis, MN, USA), with a detection limit of 0.09 pg/ml. Inter- and intra-assay coefficients of all assays were below 10%.

Gene Expression

To assess gene expression, peripheral whole blood drawn into Tempus Tubes (Tempus Blood RNA Tube, Life Technologies, Carlsbad, CA) at baseline, 30- and 120-min post-TSST on both study days was stored for up to five days at 4°C, and RNA was then extracted and isolated using Tempus Spin RNA Isolation Kits (Life Technologies, Carlsbad, CA). Aliquots were stored at −80°C until further processing. One-step RT-PCR was performed using Qiagen Quantifast Mastermix kit (Qiagen, Germantown, MD) and commercially available primers (Life Technologies) for IL-6 (00985639_m1), IL-1β (01555410_m1), RelA (00153294_m1), and IκB (00153283_m1) on a RealPlex 4S (Eppendorf, New Brunswick, NJ). The conditions for the RT cycler were 10 min at 50°C, 5 min at 95°C, and 40 cycles of 10 s at 95°C and 30 s at 60°C. Fluorescence data was collected during the extension step of the reaction using 5′ nuclease activity of FAM-labeled TaqMan probes (Life Technologies). Expression of IL-6, IL-1β, IκB, and NF-κB was normalized against expression of endogenous control GAPDH using the ΔΔCt method (ΔΔCt = ΔCt target – ΔCt control), selected because it does not respond to psychosocial stress. For each TSST, gene expression was normalized to each participant's baseline sample level.

Demographics and Anthropometrics

Participants self-reported basic demographic information. Weight and body fat measurements were taken using a Seca Supra Plus 720 column scale (Hamburg, Germany), *via* bioelectrical impedance analysis. Participant height was measured using a wall-mounted tape measure.

Statistical Analyses

All analyses were performed using SPSS 21 (IBM, Chicago, IL, USA). In preliminary analyses, Kolmogorov-Smirnov tests were computed to test for normal distribution and homogeneity of variance of all variables. Zero-order Pearson *r* correlations were used to test associations between CTQ subscale and total scores and age, sex, body fat, and depressive symptoms. Data from n = 1 participant were missing for IL-6 gene expression due to lack of qPCR amplification; n = 3 participants were missing for analyses measuring plasma IL-6 data (two for missing IL-6 data, one for exhibiting a stress response 7.9 SD above the sample mean).

To examine stress-induced changes in systemic IL-6 levels and gene expression, we used repeated-measures analysis of variance (ANOVA), with the within-subject factors "day" (day 1 vs. day 2) and "time" (pre-TSST, 30 and 120 min post TSST for each outcome variable). We then used separate ANOVAs for each TSST. For all ANOVAs, Greenhouse–Geisser correction was applied if the sphericity assumption was violated (33, 34).

To estimate IL-6 stress *reactivity*, we computed delta scores by subtracting same-day pre-stress IL-6 levels from IL-6 2-h post-TSST. To estimate IL-6 *habituation*, we subtracted the delta score derived from day 1 data from the delta score derived from day 2 data. To assess gene expression stress *reactivity*, gene expression was normalized against the baseline level of each individual participant separately for each day. To estimate gene expression *habituation*, delta scores were computed by subtracting the 30-min post-TSST expression levels on day 2 from the 30-min post-TSST expression levels on day 1. The same was done for gene expression levels at 120-min post-TSST.

To test associations between child maltreatment and gene expression responses, two approaches were used: (1) Hierarchical linear regression analyses were used to examine

associations between continuous CTQ scores (total and subscale scores) and inflammatory responses and habituation controlling for age, sex, body fat, and depressive symptoms. (2) Differences in inflammatory responses and habituation between individuals with versus without a history of child maltreatment were examined. To this end, Mann-Whitney U tests comparing distributions of TSST reactivity and TSST habituation among those scoring above vs. below CTQ cut-offs (total and subscales) were performed. Results were considered significant at $p < 0.05$. Unless otherwise indicated, reported values are untransformed means ± standard deviations (SD).

RESULTS

Preliminary Analyses

There were no significant correlations between CTQ scores (total and subscales) and body fat (all *p*s > .10). Age was marginally correlated with sexual abuse (r =.26, p =.085) but no other CTQ scores (all *p*s > .10). Depressive symptoms were significantly correlated with total CTQ scores (r =.49, p =.001), as well as with emotional abuse (r =.54, $p < .001$) and emotional neglect (r =.38, p =.01) scores. Independent samples t-tests to examine sex differences indicated marginally greater total CTQ scores [$t(44) = 1.82$, p =.076] and marginally greater sexual abuse scores [$t(27.6) = 1.18$, p =.087] among women compared to men. No other sex differences were found.

Stress Reactivity Following the TSST

Systemic Inflammation

A repeated-measures ANOVA revealed an effect of time [$F_{(1.1,44.7)} = 91.90$, $p < .001$]. To further investigate these results, separate repeated-measures ANOVAs were conducted for each day. Exposure to the TSST resulted in a significant increase in plasma IL-6 both on day 1 ($F_{1.2,50.9} = 43.7$, $p < .001$) and on day 2 ($F_{1.1,45.1} = 62.21$, $p < .001$). Further, plasma IL-6 increases from baseline to peak at 120-min post-TSST did not differ between day 1 and day 2 ($F_{2,42} = 2.57$, p =.12), suggesting that there was no habituation.

Gene Expression

Results indicated significant time effects for all gene transcripts (IL-6: $F_{2,84} = 3.1$, p =.05; IL-1β: $F_{2,86} = 8.7$, $p < .001$; NF-kB: $F_{1.7,76.6} = 8.01$, p =.001; and IkB: $F_{2,86} = 5.1$, p =.004) and significant day X time interactions for all gene transcripts (IL-6: $F_{2,84} = 7.7$, $p < .001$; IL-1β: $F_{2,88} = 12.7$, $p < .001$; NF-kB: $F_{1.8,84.6} = 4.7$, p =.01; and IkB: $F_{2,86} = 9.9$, $p < .001$). Additional repeated-measures ANOVAs revealed significant increases in all four gene transcripts in response to the TSST on day 1 (time effects: IL-6: $F_{2,88} = 10.2$, $p < .001$; IL-1β: $F_{2,88} = 10.44$, $p < .001$; NF-kB: $F_{2,88} = 10.53$, $p < .001$; and IkB: $F_{2,88} = 11.3$, $p < .001$). Significant increases in response to the TSST on day 2 were found for IL-1β and IkB ($F_{2,88} = 9.26$, $p < .001$ and $F_{2,88} = 3.20$, p =.046, respectively) but not for IL-6 and NF-kB ($F_{1.7,72.5}$ =.814, p =.43 and $F_{2,88} = 1.50$, p =.23, respectively).

Results further indicated habituation effects in response to repeated TSST exposure. Gene expression responses following TSST exposure on day 2 were significantly lower 30-min post-TSST for IL-6 [$t(44) = 4.3$, $p < .001$] and IkB [$t(45) = 4.03$, $p < .001$] in addition to marginally lower for IL-1β ($t(44) = 1.9$, p =.06). There was no significant gene expression response for NF-kB 30-min post-TSST [$t(46)$ =.84, $p > .40$]. Gene expression responses following TSST exposure on day 2 were significantly lower 120-min post-TSST for IL-1β [$t(43) = 5.8$, $p < .001$] and NF-kB [$t(45) = 2.7$, p =.009]. There was no significant gene expression response for IL-6 and IkB 120-min post-TSST [$t(42) = 1.5$, $p > .10$ and $t(43) = -.14$, $p > .80$, respectively].

Child Maltreatment and Inflammatory Responses Following Initial Stress Exposure

Total CTQ score was not associated with changes in plasma IL-6 or gene expression following the TSST (all *p*s > .10). Childhood physical neglect scores were associated with greater IL-1β mRNA response at 30-min (β =.35; p =.02; R^2 =.19) and 120-min (β =.46; p =.002; R^2 =.24) post-TSST as well as NF-kB mRNA response at 120-min post-TSST (β =.29; p =.054; R^2 =.10). See **Table 3** for full results. Other CTQ subscales were not associated with changes in gene expression following the TSST (all *p*s > .10).

These results were supported by additional analyses comparing individuals scoring above the cut-off for physical neglect to those below the cut-off. Specifically, IL-1β and NF-kB gene expression was greater 30 and 120-min post-TSST among individuals above the cut-off for physical neglect (IL-1β expression at 30 min: U = 130.00, p =.02; IL-1β expression at 120 min: U = 134.50, p =.02; NF-kB expression at 30 min: U = 167.50, p =.05; NF-kB expression at 120 min: U = 163.50, p =.04). Additionally, experiences of childhood physical neglect were associated with greater IL-6 gene expression 120-min post-TSST (U = 131.00, p =.02).

Child Maltreatment and Inflammatory Responses Following Repeated Stress Exposure

Total CTQ score was not associated with changes in plasma IL-6 levels following the TSST ($p > .10$). Physical neglect scores were associated with increases in plasma IL-6 levels in response to repeated TSST exposure on day 2 (β =.34; p =.02; R^2 =.16; see **Table 3**). Other CTQ subscales were not associated with changes in plasma IL-6 levels following repeated TSST exposure (all *p*s > .05). Testing for habituation, greater physical neglect was associated with plasma IL-6 non-habituation 120-min post-TSST (β = 0.32, p = 0.04, R^2 =.15). Mann-Whitney U-tests, however, did not confirm these differences on a between-group level (all *p*s > .10).

Total CTQ score was not associated with changes in gene expression following the TSST ($p > .10$). Physical neglect scores continued to be associated with NF-kB mRNA response 30 (β =.31; p =.04; R^2 =.08) and 120 min (β =.29; p =.07; R^2 =.03) following repeated TSST exposure. See **Table 3**. Other CTQ subscales were not associated with changes in gene expression following repeated TSST exposure (all *p*s > .10). Testing for habituation, greater physical neglect was associated with non-

TABLE 3 | Linear regressions testing the associations between childhood physical neglect and gene expression responses and plasma interleukin (IL)-6 to the TSST on day 1 and day 2.

	Time point	Gene transcripts				Plasma
		IL-6	IL-1β	NF-κB	IκB	IL-6
TSST Day 1	30 min. post	β=0.06	**β=0.35**	**β=.31**	β=0.03	β=-0.03
		p=0.69	***p*=0.02**	***p*=.04**	*p*=0.81	*p*=0.84
		R^2=0.04	**R^2=0.19**	**R^2 =.08**	R^2=0.07	R^2=0.04
	120 min. post	β=0.15	**β=0.46,**	**β=0.29**	β=0.09	β=-0.08
		p=0.36	***p*=.002**	***p*=0.07**	*p*=0.58	*p*=0.63
		R^2=0.06	**R^2=0.24**	**R^2=0.03**	R^2=0.06	R^2=0.03
TSST Day 2	30 min. post	β=-0.01	β=-0.22	β=0.11	β=-0.18	β=0.03
		p=0.94	*p*=0.16	*p*=0.48	*p*=0.24	*p*=0.85
		R^2=0.02	R^2=0.08	R^2=0.08	R^2=0.03	R^2=0.07
	120 min. post	β=-0.04	β=-0.02	**β=0.31**	β=0.28	**β=0.34**
		p=0.80	*p*=0.90	***p*=0.04**	*p*=0.07	***p*=0.02**
		R^2=0.10	R^2=0.10	**R^2=0.08**	R^2=0.03	**R^2=0.16**
Habituation	30 min. post	β=-0.06	**β=-0.47**	**β=-0.31**	**β=0.34**	β=0.22
		p=0.71	***p*=0.002**	***p*=0.03**	***p*=0.03**	*p*=0.19
		R^2=0.04	**R^2=0.24**	**R^2=0.19**	**R^2=0.18**	R^2=0.11
	120 min. post	β=-0.15	**β=-0.55**	β=0.07	β=0.11	**β=0.32,**
		p=0.35	***p*<.001**	*p*=0.69	*p*=0.49	***p*=0.04**
		R^2=0.11	**R^2=0.32**	R^2=0.09	R^2=0.07	**R^2=0.15**

Reporting results of hierarchical linear regression controlling for sex, age, body fat, depressive symptoms.
Significant associations shown in bold font.

habituation of IL-1β gene expression (β = −.47; p =.002; R^2 =.24), NF-kB gene expression (β = −.31; p =.03; R^2 =.19), and IkB gene expression (β =.34; p =.03; R^2 =.18) 30 min following TSST exposure. Additionally, greater physical neglect was associated with non-habituation of IL-1β gene expression (β = −55; p < .001; R^2 =.32) 120 min following TSST exposure.

These results were partially supported when looking at between-group differences. Changes in NF-kB gene expression were greater among those above the cut-off for physical neglect (U = 171.50, p =.047) 30 min following repeated TSST, but not 120 min following repeated exposure to the TSST (U = 174.50, p =.14). Testing for habituation, participants with a history of physical neglect were more likely to show non-habituation of IL-1β gene expression 30-min post-TSST (U = 144.00, p =.028) and marginally more likely to show non-habituation to show non-habituation of IL-1β gene expression 120-min post-TSST (U = 155.50, p =.071). Additionally, a history of physical neglect was marginally associated with non-habituation of IkB gene expression 30-min post-TSST (U = 154.50, p =.068).

Figure 1 shows changes in plasma IL-6 prior to and following the first and second TSST session, separately for those who did versus did not report having experienced physical neglect during childhood. Additionally, **Figures 2**–**5** show changes in all four gene transcripts.

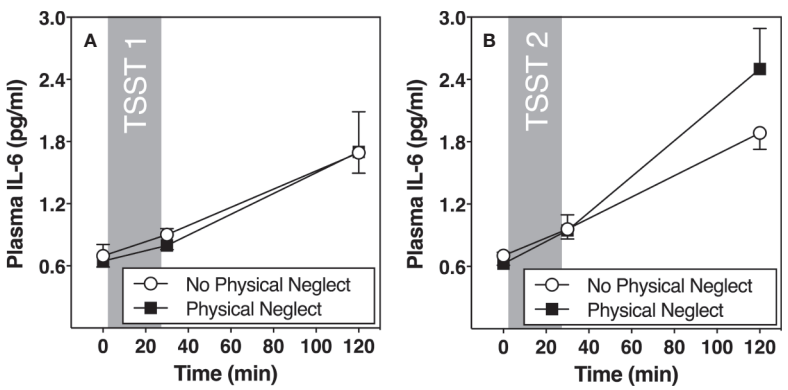

FIGURE 1 | (A) Means and standard errors of the mean (SEM) of plasma IL-6 response to TSST1 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect; **(B)** Means and standard errors of the mean (SEM) of plasma IL-6 response to TSST2 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect.

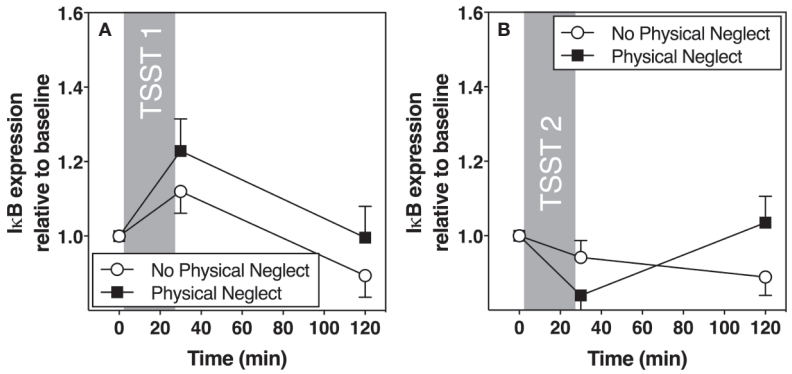

FIGURE 2 | (A) Means and standard errors of the mean (SEM) of I-kB gene expression response to TSST1 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect; **(B)** Means and standard errors of the mean (SEM) of I-KB gene expression response to TSST2 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect.

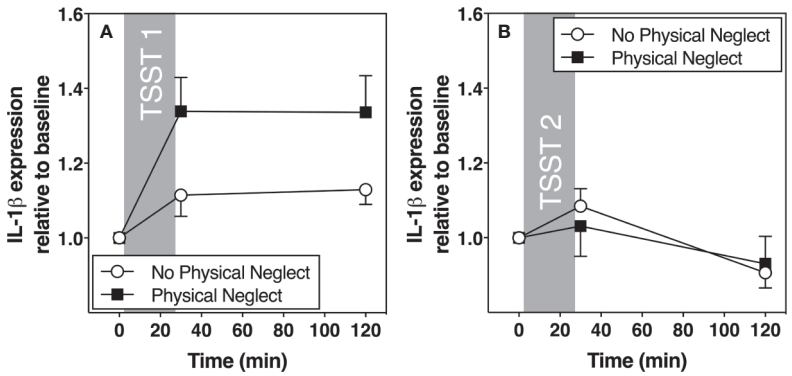

FIGURE 3 | (A) Means and standard errors of the mean (SEM) of IL-1β gene expression response to TSST1 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect; **(B)** Means and standard errors of the mean (SEM) of IL-1 β gene expression response to TSST2 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect.

DISCUSSION

We set out to investigate the association between child maltreatment history and plasma inflammatory as well as inflammatory gene expression reactivity in response to initial and repeated exposure to an acute, laboratory-based psychosocial stressor. Our hypotheses were partially supported. Specifically, no effect of total CTQ scores was found on either plasma inflammatory and inflammatory gene expression responses following initial or repeated TSST exposure, counter to previous reports linking broad indices of having experienced any child maltreatment to increased acute stress responses (15, 20, 35). Additional exploratory analyses, however, found consistent evidence of an association between a history of childhood physical neglect and multiple of the examined outcomes. History of physical neglect was associated with greater IL-1β and NF-kB expression to initial acute stress exposure and reduced habituation to repeated stress with respect to plasma IL-6 levels and inflammatory gene expression.

Interestingly, our results reveal two different response patterns of peripheral inflammatory changes after acute stress. Gene expression data based on all four transcripts suggest rapid increases following acute stress—peaking 30-min post-TSST—as well as strong habituation. Conversely, levels of plasma IL-6 showed evidence of a much slower increase with a peak at or after 120-min post-TSST and no habituation. These differences in kinetics are most likely explained by the fact that these outcomes capture different stages of the inflammatory response following acute stress. Changes in gene expression responses are most likely the result of rapid signaling *via* adrenergic receptor processes to the cell (16). Additionally, a redistribution of immune cells following acute stress may partly explain the observed increases (36). Delayed peaks of plasma IL-6, on the other hand, as well as lack of habituation, have been reported previously, including by us and other groups (37, 38). Plasma IL-6 is thought to stem from a number of tissue sources, such as adipose

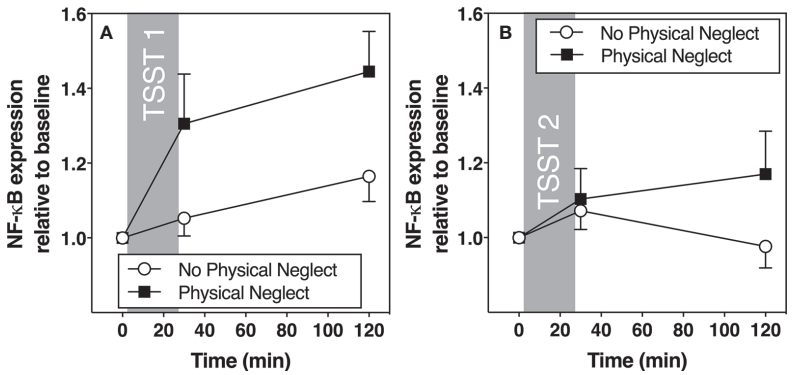

FIGURE 4 | (A) Means and standard errors of the mean (SEM) of NF-kB gene expression response to TSST1 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect; **(B)** Means and standard errors of the mean (SEM) of NF-KB gene expression response to TSST2 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect.

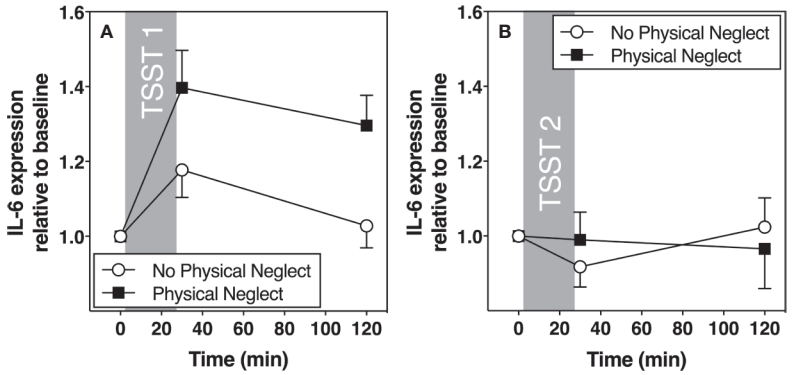

FIGURE 5 | (A) Means and standard errors of the mean (SEM) of IL-6 gene expression response to TSST1 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect; **(B)** Means and standard errors of the mean (SEM) of IL-6 gene expression response to TSST2 at baseline as well as 30- and 120-min post-TSST in those with and without childhood physical neglect.

tissue and endothelial cells, in addition to immune cells [e.g., (39, 40)], and due to this somewhat unclear mix of tissue origins, plasma increases are likely the result of a different, and slower mechanism of the inflammatory acute stress response than the mechanism observed when testing gene expression. Future research, however, will need to examine in greater detail why gene expression effects show strong signs of habituation, whereas plasma IL-6 responses do not show signs of habitation and may, in fact, show signs of sensitization following repeated exposure to acute stress [see, e.g., (21)].

Although the present results are based on exposure to repeated acute stress within the context of a highly controlled lab environment, they have implications for the long-term health of those exposed to child maltreatment experiences in their youth. The biological embedding model (41, 42) posits that early adversity programs cells of the innate immune system to respond to challenge in a pro-inflammatory fashion. Although likely adaptive in the short-term, the resulting more aggressive immune system response may result in a pro-inflammatory immune profile that confers a long-term increase in disease risk. Much more research is needed before findings such as these can be translated into medical interventions targeting child maltreatment survivors. Nonetheless, understanding how early experiences, such as child maltreatment, alter key aspects of the immune system response that have the potential to increase individuals' risk for chronic diseases of aging represents an important first step in that direction.

It is unclear why physical neglect stands out as a powerful differentiator of inflammatory and gene expression responses to acute psychosocial stressors. Interpretation of these findings is further hampered by the lack of prior research on the physiological consequences of exposure to childhood neglect among humans. We note that these effects were found even though all analyses were adjusted for depressive symptoms. Animal models of deprivation, not unlike physical neglect in humans, have previously been linked to different, though related, adverse outcomes including anxiety, behavioral despair, attenuated HPA axis reactivity, and freezing in response to aggression from

other animals (43–45). Alternatively, although speculative, it is possible that physical neglect in particular reflects an overall home environment in which individuals were exposed to pervasive and chronic stressors, such as unsanitary housing, lack of access to healthy foods, etc. Ultimately, however, more work among humans examining long-term physiological consequences of childhood neglect is needed to better answer these question and to put the current findings into context.

This study builds upon previous findings by documenting reduced habituation of stress-induced expression of selected inflammatory genes and plasma inflammatory molecules in individuals with childhood physical neglect. This highlights the importance of considering the acute stress response following repeated exposure to stressors which more closely mimics stress exposure during everyday life. Given that individuals with a child maltreatment history may be especially likely to experience frequent stressors during adulthood, this combined exposure may partly explain poorer health among those with a history of child maltreatment. For example, the effects of altered inflammatory responses following acute stressors may become amplified by greater incidences of subsequent stressful life experiences.

Finally, although no association was found between history of physical neglect and plasma IL-6 reactivity to TSST exposure, we did observe differences in habituation to repeated stress. Thus, it is possible that within this limited-adversity sample, we were able to capture evidence of altered adaptation to repeated stress more readily than reactivity to one-time acute stress exposure.

This study has several important strengths. Our sample included both men and women and we were able to examine the effects of not only one-time but repeated exposure to a well-established laboratory-based acute psychosocial stressor. Additionally, we were able to explore associations across different subtypes of maltreatment, thereby adding to the extremely sparse existing literature focusing on the physical health consequences of childhood physical neglect.

Nonetheless, some limitations warrant discussion. Child maltreatment history was assessed using a retrospective self-report questionnaire which introduces the possibility of recall bias of childhood exposure to such experiences. However, the CTQ is a widely used instrument and examinations of the effects of child maltreatment experiences outside of the scope of expensive, long-term prospective studies are bound to suffer from assessment challenges as many cases of child maltreatment go unreported even in official records. Somewhat encouragingly, retrospective self-reports may include more false negative than false positive reports, suggesting that associations reported here may be conservative (46). Relatedly, participants in this study were generally healthy and reported overall low to moderate levels of child maltreatment experiences which may explain the absence of hypothesized associations between sexual and physical abuse history and acute inflammatory stress reactivity. Yet, the fact that consistent effects of physical neglect exposure were found even among a healthy sample of adults without psychiatric or medical conditions, may speak to the strength of this association and warrants further investigation. Additionally, existing research supports the importance of investigating possible effects of low severity child maltreatment exposures as it has been shown that even mere investigations of ultimately unsubstantiated cases of child maltreatment confer an increased risk of later adverse health outcomes (47). Regardless, future research should attempt to replicate these findings in samples also including individuals with exposure to more severe child maltreatment experiences. Finally, our exploratory analyses resulted in a larger number of statistical models being run, potentially increasing concerns regarding type one error findings. This is mitigated by the fact that significant associations were consistently found for the effect of childhood physical neglect history across a number of related outcomes as well as consistently in the same and in the hypothesized direction, which greatly reduces the likelihood that these findings represent false positive findings.

Taken together, these findings provide preliminary, yet intriguing, evidence that childhood physical neglect, even at relatively moderate levels, has the potential to result in exaggerated plasma inflammatory and inflammatory gene expression responses to acute psychosocial stress. Furthermore, by adding the examination of these physiological responses following repeated exposure to the same psychosocial stressor, we were able to examine differences in habituation to repeated psychosocial stress which has important implications for individuals with a child maltreatment history.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Brandeis University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NR, YK, CM, and MT planned and conceptualized the study. MT, DS, LH, XC, DW, and DG collected data. YK, CM, HS, and NR analyzed the data. HS, YK, CM, and NR wrote the manuscript. All authors reviewed the manuscript file.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Early Life Adversities and Borderline Intellectual Functioning Negatively Impact Limbic System Connectivity in Childhood: A Connectomics-Based Study

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Early life adversity (ELA) in childhood is a major risk factor for borderline intellectual functioning (BIF). BIF affects both adaptive and intellectual abilities, commonly leading to school failure and to an increased risk to develop mental and social problems in the adulthood. This study aimed to investigate the neurobiological underpinnings of ELA associated with BIF in terms of global topological organization and structural connectivity and their relation with intellectual functioning. BIF (N=32) and age-matched typical development (TD, N=14) children were evaluated for intelligence quotient (IQ), behavioral competencies, and ELA. Children underwent an anatomical and diffusion-weighted MR imaging (DWI) protocol. Global brain topological organization was assessed measuring segregation and integration indexes. Moreover, structural matrices, measuring normalized number of fibers (NFn), were compared between the 2 groups using network-based statistics. Finally, a linear regression model was used to explore the relationship between network parameters and clinical measures. Results showed increased behavioral difficulties and ELA, together with decreased network integration in BIF children. Moreover, significantly lower NFn was observed in the BIF group (p=.039) in a sub-network comprising anterior and posterior cingulate, the pericallosal sulcus, the orbital frontal areas, amygdala, basal ganglia, the accumbens nucleus, and the hippocampus. Linear regression showed that NFn significantly predicted IQ (p<.0001). This study demonstrated that ELA in children with BIF is associated with a decreased information integration at the global level, and with an altered structural connectivity within the limbic system strictly related to the intellectual functioning.

Keywords: early life adversity, borderline intellectual functioning, limbic system, connectomics, graph analysis

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INTRODUCTION

There is compelling evidence that Early Life Adversities (ELA) in childhood, such as low socio-economic status (SES), maltreatment, neglect, and high levels of parental/family stress, are major risk factors for mental health disorders (1, 2). Moreover, several neuroimaging studies investigating the impact of ELA revealed that low SES, maltreatment and neglect, if experienced during childhood, are associated with abnormal brain function and development in several regions, particularly within the limbic system (3–5). These data have been considered as part of the biological substrate of the "latent vulnerability" (6) according to which the alterations observed at the structural and functional level in several neurobiological systems reflect the (mal)adaptation to neglectful and/or abusive early environments. These changes are likely to be beneficial within the maladaptive context but represent a long-term cost for the subject, thus increasing vulnerability to future stressors (6).

In this context, a particularly vulnerable population is represented by children with borderline intellectual functioning (BIF). BIF is a neuropsychiatric condition characterized by an intelligence quotient (IQ) in the borderline range (70 to 85) associated with adaptive difficulties in social participation (7, 8). The major risk factor for the development of BIF is represented by ELA (9–12). In primary school age, BIF is characterized by limitations in social (13, 14), emotional and behavioral capacities (15, 16). According to recent studies, the prevalence of BIF has been established to be as high as 7 to 12% (7, 17). Children with BIF have a risk as adults to develop mental health problems (e.g., antisocial personality disorder, depression, psychosis, suicide and substance abuse), physical problems and poverty compared to people with average or above average IQ (18–20). Moreover, the presence of BIF negatively impacts the prognosis of all psychiatric diseases (21). Taken together, these data show that BIF is a highly relevant condition for the prognosis and treatment of neuropsychiatric disorders in childhood.

Recently, neuropsychiatric disorders are increasingly being investigated at the level of distributed brain networks rather than in the context of individual brain regions (22, 23). The network-based approach to the study of brain connectivity, so-called brain connectomics, allows the investigation of the topology of the brain as a network. According to graph theory, the brain can be viewed as a complex network constituted of nodes, i.e., the cortical and subcortical gray matter (GM) structures, with pairs of nodes connected by edges, i.e., the white matter (WM) fibers that connect them. This framework allows for the investigation of several properties of the network that define its efficacy and complexity. According to Friston et al. (24) there are two fundamental principles of brain organization: functional specialization (segregation) and functional integration according to which complex behaviors derive from the integration of functionally specialized areas that are highly interconnected to form clusters and modules. Graph theory enables the measurement of the metrics exploring this type of organization from a topological point of view.

BIF is a condition characterized by a heterogeneous behavioral phenotype and therefore it is reasonable to think that a distributed network is involved in its clinical manifestations.

The aim of this study was then to investigate the brain network connectivity of children exposed to adverse social environments showing a BIF and its relationship to intellectual functioning. The answer to these questions can have a great impact for the planning of appropriate rehabilitative intervention to prevent the many risks this population faces in the adult age.

We therefore created an *ad hoc* checklist, the environmental stress check list (ESCL), to assess the adversity that children with BIF were exposed to and compared children with BIF with children with typical development (TD) in terms of brain connectivity and topological organization. Finally, to explore the relationship between intellectual functioning and clinical, environmental, and brain connectivity indices, a linear regression approach was used.

METHODS AND MATERIALS

Participants

Forty-two children with BIF associated with significant ELA (see later for more details) were recruited from the Child and Adolescent Neuropsychiatry Unit of IRCCS Don Carlo Gnocchi Foundation and the ASST S. Paolo and S. Carlo Hospital.

Inclusion criteria were: (1) age range comprised between 6 to 11 years old; (2) attendance of a primary mainstream school; (3) a Full Scale Intelligence Quotient (FSIQ) score ranging from 70 to 85 determined with the Wechsler Intelligence Scale for Children-III (WISC-III) (25).

A group of eighteen age and sex-matched TD children with a negative history for neurodevelopmental, behavioral or emotional disorders and a FSIQ >85 was also included in the study. The recruitment of the healthy controls was made through the advertisement of the study among the workers of our Institute.

All subjects underwent a clinical evaluation with a detailed medical history of the child and of his/her family, and clinical observations. Moreover, all subjects underwent also a CBCL evaluation (26). All neuropsychiatric diagnosis were made according to the DSM 5 criteria.

To exclude children with BIF due to biological and/or genetic causes, the presence of any of the following represented an exclusion criteria: (1) major neuropsychiatric disorders (such as ADHD, and autism spectrum disorder); (2) neurological conditions such as epilepsy, traumatic brain injury, brain malformation, infectious disease involving the central nervous system and perinatal complications such as prematurity or other adverse events; (3) systemic diseases such as diabetes or dysimmune disorders, genetic syndromes such as Down syndrome or Fragile X syndrome. Furthermore, a positive history for psychoactive drugs, particularly referring to current or past use of psychostimulants, neuroleptics, antidepressants, benzodiazepines, and antiepileptic drugs were also considered exclusion criteria.

All children underwent a neuropsychological evaluation including: the WISC-III (25); the Child Behavioral Checklist (CBCL 6-18) (26); the SES (27); the ESCL, an *ad hoc* developed

check list to explore the environmental stress the children were exposed to (See **Supplementary Table S1**). The ESCL comprised a listing of the V-codes from DSM-5, and Z-codes from ICD-10, exploring Relational, Neglect, Physical, Sexual, and/or Psychological Abuse, Educational and Occupational, Housing and Economic, Social Exclusion, or Rejection Problems, plus the presence of the following three conditions: social services intervention, major psychiatric diagnosis, and/or substance abuse within the family members. The presence of each condition and its relevance for the clinical manifestations was considered and a 0 (absence) to 1 (presence) score was attributed to each item. The ESCL total score could range from 0 to 24. The considered conditions were not weighted for their severity, thus in general higher scores do not represent a more adverse environment.

All subjects underwent a magnetic resonance imaging (MRI) evaluation (see MRI Acquisition section).

The Study was approved by the Ethics Committees of the Don Gnocchi Foundation and of the ASST S. Paolo and S. Carlo Hospital. All parents signed a written informed consent at the first meeting.

MRI Acquisition

MRI was performed on a 1.5 T Siemens Magnetom Avanto (Erlangen, Germany) scanner equipped with a 12-channels head coil. The acquisition included: (1) a 3D T1-weighted Magnetization Prepared Rapid Gradient-Echo (MPRAGE) image, (repetition time (TR)/echo time (TE)=1900/3.37 ms, Filed of View (FoV) = 192x256 mm2, voxel size = 1 mm isotropic, 176 axial slices); (2) a diffusion-weighted (DW) EPI image along 30 directions with b-value=1,000 s/mm2 and one without diffusion weighting (TR/TE = 6,700/100 ms, FoV = 200x200 mm2, voxel size 1.6x1.6x2.5 mm3, 40 axial slices, two runs); (3) two conventional anatomical sequences (axial PD/T2 and coronal FLAIR) to exclude gross brain abnormalities.

MRI Data Analysis

The 3D-T1 images were segmented and parcellated using FreeSurfer version 5.3[1] into 148 cortical areas (74 for each hemisphere) according to the Destrieux atlas (28). Furthermore, the FreeSurfer automatic labeling process was used to extract seven subcortical regions per hemisphere (thalamus, caudate, putamen, pallidum, and nucleus accumbens, amygdala and hippocampus) and the brain stem for a total of 163 parcels. The quality of recon-all parcellation was assessed in each subject according to ENIGMA guidelines[2] for cortical and subcortical areas.

Using FMRIB's Software Library tools[3], the DW images were corrected for eddy current distortion (29). The motion evaluation was performed by checking the relative movement estimated by eddy toolbox and excluding subjects exciding a threshold fixed to 0.5. Then, using the FSL DTIFIT toolbox[4] the tensor was estimated for each voxel. The cortical/subcortical parcels were registered to the DW space using the FSL flirt tool (30). Finally, the WM tracts connecting each pair of registered cortical and subcortical parcels (nodes) were reconstructed with TrackVis software[5].

The connectivity matrices were derived by computing the edges as the number of the reconstructed fibers normalized by the sum of the nodes volumes (NFn) in order to consider the effect of anatomical variability. The matrices were successively employed both for graph and network-based analyses as explained in details in the following sections.

Graph-Based Analysis

The connectivity matrices were thresholded and binarized. The matrices were thresholded in a way that at least 1/3 of TD children shared the same connections. In order to investigate the topological organization, whole brain network metrics of segregation and integration were derived (31). Specifically, average clustering coefficient (CC), characteristic path length (CPL), and global efficiency (GE) were computed for each subject by means of the Brain Connectivity Toolbox (brain-connectivity-toolbox.net). Furthermore, the density of the matrices was extracted (see **Table 1**). The resulting indices were compared between the two groups by means of a Mann-Whitney test. All the computed measures (except for the clustering coefficient which was derived for each node and then averaged to have a single value for each matrix, i.e., average clustering coefficient) are global ones which means that a single value is derived for each subject. Therefore, the Bonferroni correction was applied considering the number of indices computed.

Network-Based Analysis

Potential group differences were computed using the Network-Based Statistics toolbox (NBS) (32), using an ANCOVA design with age and sex as covariates. The threshold used to identify connections was set to 3.1 (p=.0017), with family-wise error (FWE)-correction using permutation testing (10,000 permutations, p=.05). The results were visualized using a circular representations [connectograms, http://circos.ca/ (33)], according to (34, 35). Finally, to correlate the results deriving from the NBS analysis with the clinical data, the cluster strength (CS) was calculated as the mean strength (weights average) of the sub-network of significant difference between the two groups. A partial correlation (Spearman) with the clinical variables, FSIQ, SES, CBCL, ESCL, and the CS, with age and sex as covariates, was then performed. The variables showing a significant correlation were considered as independent variables in a regression model predicting the FSIQ score. All statistical analyses were performed by means of SPSS (Version 25; IBM, Armonk, New York) software.

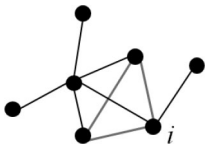
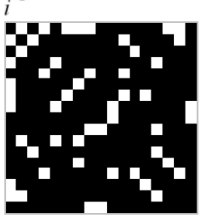
RESULTS

Sample and Clinical Assessment

Due to excessive head movement during the MRI evaluation, 10 children with BIF and 4 TD were excluded from the data

[1] https://surfer.nmr.mgh.harvard.edu/
[2] http://enigma.ini.usc.edu/enigma-vis/
[3] FSL; http://www.fmrib.ox.ac.uk/fsl
[4] https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FDT
[5] http://trackvis.org

TABLE 1 | Graph indices description.

Index	Graphical Representation	Mathematical Expression	Definition
Average Clustering coefficient (CC)		$CC = \frac{1}{n}\sum_{i \in N}\frac{2t_i}{k_i(k_i-1)}$	The Average Clustering coefficient (CC) is a measure of segregation expressing the degree of connection of the nodes neighborhood.
Characteristic path length (CPL)		$CPL = \frac{1}{n}\sum_{i \in N}\frac{\sum_{j \in N, j \neq i} d_{ij}}{n-1}$	The characteristic path length (CPL) is a measure of integration, expressing the average shortest path between nodes pair.
Global Efficiency (GE)		$GE = \frac{1}{n}\sum_{i \in N}\frac{\sum_{j \in N, j \neq i} d_{ij}^{-1}}{n-1}$	The global efficiency (GE) is a measure of how efficiently the information travel through the whole network. It is the average inverse of the characteristic path length.
Density (D)		$D = \frac{K}{(n^2-n)/2}$	The density (D) is a measure of sparsity of the matrix: it represents the number of actual connections (K) with respect to the number of possible connections (n).

The table shows an overview of the computed global indices (31): specifically for each index a graphical, mathematical and theoretical description is provided. Legend: t=number of triangles of a node neighborhood; d=distance between a pair of nodes; k=number of actual connections (edges different from zero); n=number of possible connections.

analyses, and thus the final sample consisted of thirty-two BIF and fourteen TD children.

Among the 32 children with BIF, 25 had an Adjustment Disorder, 4 had a Generalized Anxiety Disorder and 1 had a Disruptive, Impulse-Control, and Conduct Disorder. Moreover, in 14 children a Specific Learning Disorder was associated, in 14 there was a history of Language Development Disorder.

Demographic data relative to the 32 BIF and 14 TD children are shown in **Table 2**. No significant differences were found for age, sex, and SES, while the CBCL (p=0.005), the ESCL and as expected the FSIQ (p<0.0001) were significantly different between the two groups (see **Table S2** for ESCL detailed score).

Graph-Based Analysis Results

To determine the topological organization of the brain in the two groups, whole brain metrics were derived globally. Specifically, the CC, the CPL, and the GE indices were calculated for each subject and compared between the two groups. The density (D) of the matrices was also computed. **Table 3** shows results of the Mann Whitney analysis showing significantly increased CPL (p<0.001), and significantly reduced GE (p=0.001) and D (p=0.006) in the group of children with BIF. The CC index, measuring the network segregation, was not different between the two groups.

Network-Based Analysis Results

The NBS analysis comparing the two groups of children in terms of structural connectivity (number of fibers normalized for the volume of the GM parcels) at the network level revealed a sub-network comprising 67 edges and 51 nodes for which the group of children with BIF had a significantly lower NFn compared to

TABLE 2 | Demographic variables.

	TD (n=14)	BIF (n=32)	p-value
Age in yrs, median (IQR)	9.2 (8.5 - 9.6)	8.6 (8.2 - 9.9)	0.543[a]
Male n,(%)	7 (50%)	17 (53%)	0.9[b]
FSIQ, median (IQR)	119 (111.5 - 121.5)	80 (75 - 84)	**<0.0001[a]**
CBCL, median (IQR)	49.5 (41 - 53)	59 (52 - 66.5)	**0.005[a]**
SES, median (IQR)	25 (25 - 48.8)	23.5 (16.6 - 31.4)	0.119[a]
ESCL, median (IQR)	0.0 (0.0 – 1.0)	2.0 (1.0 – 4.0)	**<0.0001[a]**

IQR, Interquartile range. [a]Non-parametric (Mann-Whitney test), [b]Chi-squared test. FSIQ, Full Scale Intelligence Quotient; CBCL,Child Behavior Checklist, SES, Socio-Economic Status; ESCL, Environmental Stress Check List. The correction for multiple comparisons was implemented with the Bonferroni Correction, setting the significance threshold at p ≤ 0.0125. Significant p-values are highlighted in bold.

TABLE 3 | Graph-based indexes.

	TD (n=14)	BIF (n=32)	p-value
Clustering Coefficient, median (IQR)	0.75 (0.74 - 0.75)	0.74 (0.74 - 0.76)	0.252
Characteristic Path Length, median (IQR)	1.56 (1.54 - 1.56)	1.58 (1.57 - 1.61)	**0.0002**
Global Efficiency, median (IQR)	0.72 (0.71 - 0.73)	0.71 (0.70 - 0.72)	**0.001**
Density, median (IQR)	0.69 (0.64 - 0.69)	0.63 (0.61 - 0.65)	**0.006**

Global indices derived from the thresholded and binarized whole brain network matrices for the two groups and their comparison (Non-parametric, Mann-Whitney tests). IQR, Interquartile range. The correction for multiple comparisons was implemented with the Bonferroni Correction, setting the significance threshold at p ≤ 0.0125. Significant p-values are highlighted in bold.

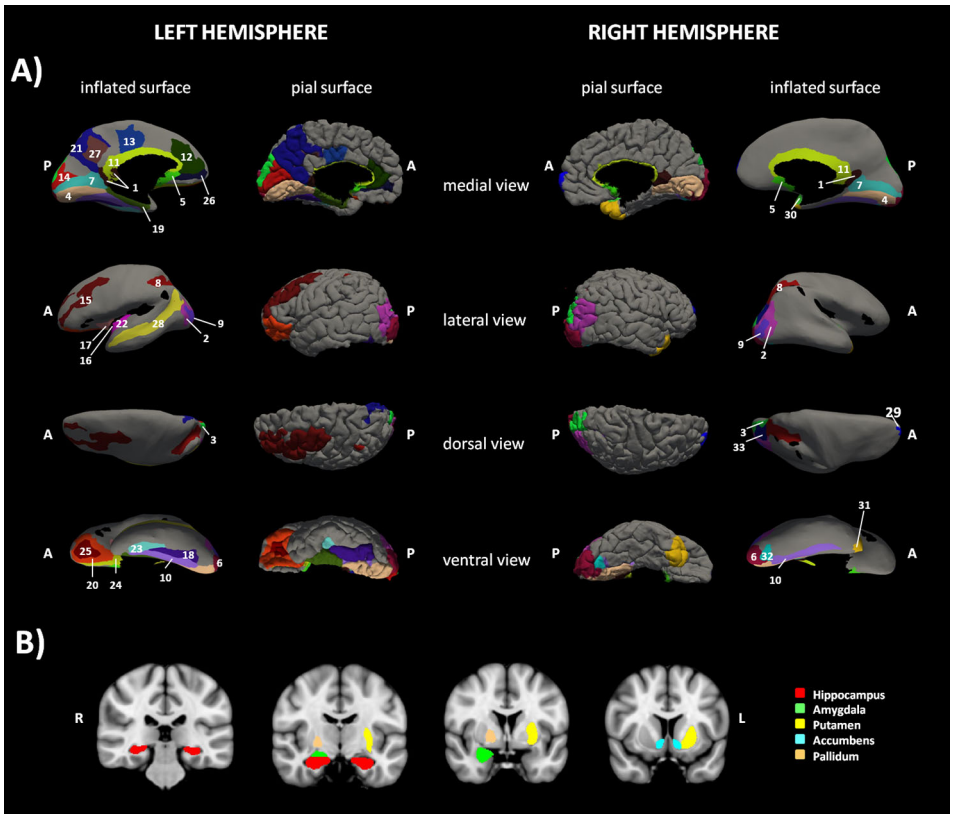


FIGURE 1 | Sub-network cortical/subcortical labels. The figure shows the sub-network of significant difference between the two groups of children obtained with network-based statistic (NBS). **(A)** shows the cortical nodes while **(B)** shows basal ganglia belonging to the sub-network. 1=L and R vPCC G, 2=L and R Middle occipital G, 3=L and R Superior occipital G, 4=L and R Lingual part of the medial occipito-temporal G, 5=L and R Subcallosal G, 6=L and R Occipital Pole, 7=L and R Calcarine S, 8= L and R Intraparietal and tansverse parietal S, 9=L and R Middle Occipital and Lunatus S, 10=L and R Collateral and Lingual S, 11=L and R Pericallosal S, 12=L ACC G and S, 13=L pMCC G and S. 14=L Cuneus G, 15=L Middle Frontal G, 16= L Long Insular G and central S of the Insula, 17=L Short Insular G, 18=L Fusiform G, 19=L Parahippocampal part of the medial occipito-temporal G, 20=L Orbital G, 21=Precuneus G, 22= L Inferior Segment of the Circular S of the Insula, 23=L Anterior Transverse Collateral S, 24=L Medial Orbital (Olfactory) S, 25= L Orbital (H Shaped) S, 26=L Suborbital S, 27=L Subparietal S, 28=L Superior Temporal S, 29=R Transverse Frontopolar G and S, 30=R Planum polare of the Superior Temporal G, 31=R Temporal Pole, 32=R Posterior Transverse Collateral S, 33= R Superior occipital and Transverse Occipital S. G=Gyrus/i, S=Sulcus/i; R=right hemisphere, L=left hemisphere, ACC=anterior cingulate cortex, pMCC=middle posterior cingulate cortex, vPCC=ventral part of the posterior cingulate cortex.

the TD group (p=.045 FWE-corrected, see **Supplementary Table S3**). No significant differences were found in the opposite comparison, TD versus BIF children. The nodes identifying the sub-network comprised the posterior ventral cingulate (vPCC), the striate and extrastriate occipito-temporal cortices, the pericallosal sulcus, the subcallosal gyrus, the hippocampus, the accumbens nucleus, and the intraparietal sulcus, bilaterally. The orbital cortex, the insula, the putamen, and the anterior and middle posterior cingulate were involved on the left side while the amygdala, the pallidum, and the superior temporal gyrus (planum polare) on the right side (see **Figure 1**).

In **Figure 2**, the 67 edges connecting the nodes of the identified sub-network are represented on a connectogram (see **Supplementary Table S3** for a complete listing).

The partial correlation analysis between the clinical variables FSIQ, SES CBCL total score, ESCL and the CS, with age and sex as covariates, resulted significant for the FSIQ and the CS (r= 0.71; p<0.0001), FSIQ, and CBCL (r=-0.47; p=0.003) and FSIQ and ESCL (r=-0.45, p=0.004). The correlation between FSIQ and SES did not survive statistical threshold (r=0.24, p=0.144) According to the correlation results, the linear regression model comprised FSIQ as the dependent variable and CS, CBCL, and ESCL as independent variables. Results revealed that only the CS had a significant predictive value for the FSIQ (R2 = 0.58; beta=0.6; t=5.0; p<0.0001).

DISCUSSION

Our study focused on the neurobiological underpinnings of ELA associated with BIF. To this purpose we investigated brain network topological organization and connectivity in 32 children with BIF and 14 age-matched TD children. All children were clinically characterized for exposure to environmental stress, intellectual functioning and behavioral characteristics.

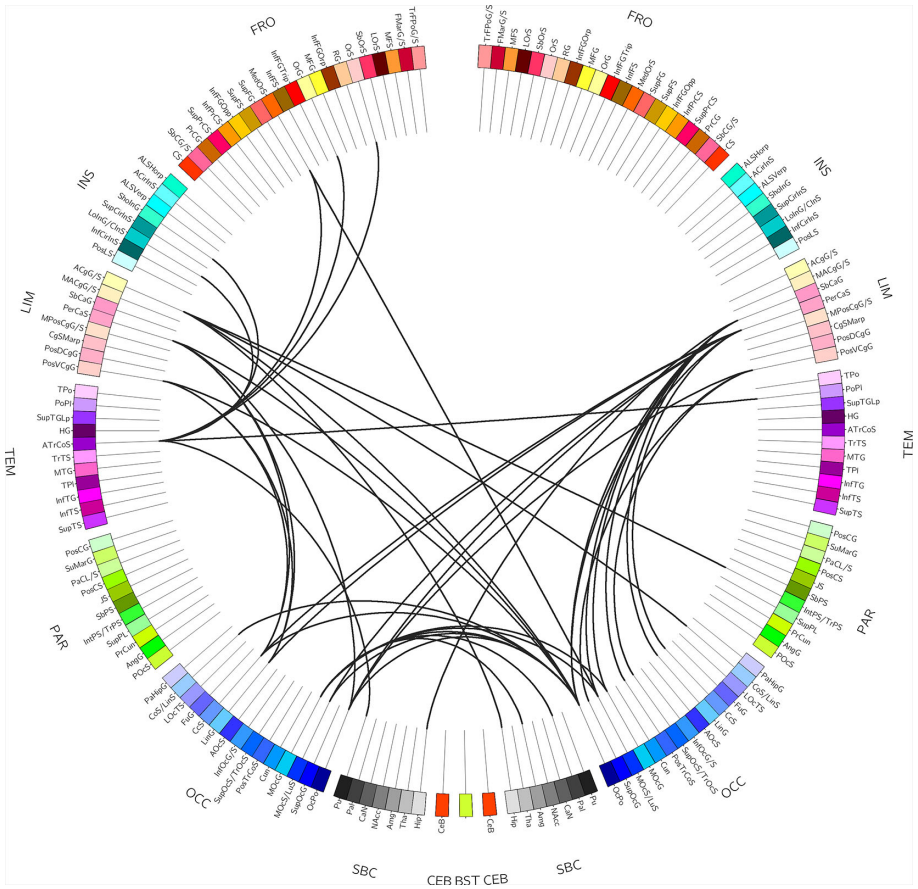


FIGURE 2 | Connectogram-based edges representation. The figure shows the circular representation of the edges belonging to the sub-network of significant difference derived from network-based statistic (NBS). For a complete listing of the nodes involved see **Supplementary Table 2**.

The interest in children with BIF condition is in line with the emerging concept of a preventative psychiatry oriented towards the development early intervention models for mental health making it a priority to intervene in the developmental period. This approach can benefit from the understanding of the neurocognitive mechanisms involved in psychiatric disorders that share common ethiopatogenetic mechanisms rather than conventional diagnostic boundaries (36). Children with BIF indeed represent a population highly vulnerable to the development of major psychiatric conditions as adults (19, 21) with environment-related etiopathogenetic factors (9–12).

The results of this study showed that the children with BIF were characterized by significantly more environmental stressful elements and behavioral difficulties compared to the TD children. The major sources of stress in the group of children with BIF were related to educational problems, family disaggregation and/or the intervention of social services for impossibility of the parents to cope. Moreover, in few cases there was a history of parents' drug abuse, or abandonment of children by the father, or father in prison. Some children grew up away from their parents. In few cases, the low SES resulted in inadequate housing in a way that was considered relevant to the diagnosis. This is in line with data from recent studies, one of which investigated a very large cohort (14,000 children) and showed that children from low SES families scored on average 6 IQ points lower at age 2 than children from high SES families; by age 16, this difference had almost tripled (37). Moreover, it has been demonstrated that low SES affects both learning abilities and brain development in regions critical for memory and emotion regulation such as the hippocampus and the amygdala (3). In our study, the SES was not statistically different between the two groups of children but the BIF group was exposed to significantly greater environmental stress. Since previous neuroimaging studies have demonstrated that maltreatment and/or neglect in childhood can impact brain development (4, 38–40) our study investigated the neural bases of the BIF condition. In a previous study from our group, abnormal brain development of the parahippocampal, temporal and sensory-motor cortices in children with BIF was found with a single brain region approach (41). In this study, we used a brain connectomics approach, and the whole brain topological

organization was investigated to capture indices of global organization by using a graph-based analysis. Results showed significant differences between the two groups in the CPL and in the GE. Both indices explore the level of integration of the information coming from different brain structures and the observed differences indicate lower levels of integration in the group of children with BIF. The lower level of integration reasonably reflects the broad range of cognitive and behavioral difficulties observed in children with BIF ranging from specific learning disorder to difficulties in higher order functions such as executive functions, planning, inhibition, attention, and behavior (7). These data are in line with a study on healthy adult subjects showing that higher intelligence scores corresponded to a shorter CPL and a higher GE of the networks, indicating a more efficient parallel information transfer in the brain (42).

Beside the graph-based analyses, we further investigated structural connectivity using a network-based approach. Results revealed significant between-group differences in a sub-network connecting several cortical and subcortical areas, mostly related to the limbic system. Specifically, the anterior and posterior cingulate cortices, the hippocampus and the parahippocampus, the pallidum, the putamen, and the accumbens nucleus, the subcallosal and pericallosal cortices, the frontal-orbital regions, and the extra-striate visual cortices were part of the network. To our knowledge this is the first report of the involvement of the limbic system connectivity in children exposed to ELA showing a BIF and is in line with consistent data from neuroimaging studies showing that abuse, maltreatment, and neglect in childhood are associated with specific epigenetic and neural signatures related to long lasting structural and functional changes in brain areas belonging to the limbic system (4, 36, 43) together with alteration in the structural connectivity at the network level (38). In particular, it has been shown that adults who experienced maltreatment during childhood show hyper-responsiveness of the amygdala to fearful stimuli (44, 45), even during pre-attentive conditions (46, 47), hypo-reactivity of the hippocampus to pleasant autobiographical stimuli, and hyper-reactivity during unpleasant stimuli (46), and abnormal reactivity to reward in the nucleus accumbens (48). Moreover, at the morphometric level reduced cortical thickness in the anterior cingulate, superior frontal gyrus, and orbitofrontal cortex, reduced cortical surface area in the left middle temporal area and lingual gyrus, and gyrification deficits in the lingual gyrus and the insula were demonstrated (4). Finally, altered structural brain network topology was found at the global and lobar level in maltreated children with normal IQ, with significant reductions of the connectivity strength and increment in the CPL, both related to neural integration capacity (38). These data thus showed functional and structural alterations in the neurocognitive systems involved in fear – emotion regulation, motivation – reward, and learning processes, which have been considered a sort of early developed adaptive calibration of these systems to adverse environments. In turn, these changes may represent a "latent vulnerability" to future stressors associated with an increased risk of developing mental health disorders later in life (6).

In the present study, the clinical population involved children exposed to several environmental stressors associated with BIF in the presence of clinical manifestations ranging from neurodevelopmental disorders such as specific learning disorder, language, and movement development disorders to adjustment or behavioral or anxiety disorders. Therefore, BIF cannot be considered a latent condition but a clinically manifest one that necessitates immediate interventions. Moreover, the involvement of the whole limbic system is in line with the clinical manifestations of these children ranging from the emotion regulation/behavioral problems, difficulty in inhibiting impulsive responses, and the motivational problems (8) and with the poor prognosis in terms of risk to develop psychiatric disorders in the adult age (18–20). The limbic system has a pivotal role in all these aspects (49).

To investigate the causal relationship between the IQ and the clinical, neural, and environmental aspects a linear regression approach was used. Results showed that the cluster strength of the altered network was a predictor of the IQ of the children, while the clinical (CBCL) and environmental (ESCL) variables were not. These data demonstrate a strict relationship only between the network connectivity and the intellectual functioning of our children. This could be due either to a non-linear relation between the other variables or to the absence of such a relationship. Moreover, the ESCL was not a weighted measure and thus did not reflect the severity of the environmental condition. In the interpretation of these results, the link between the BIF condition with several neuropsychiatric conditions has to be considered. Indeed, children with BIF are characterized also by their difficulties in adaptive abilities. For this reason, the association between the network connectivity and the IQ is likely to be mediated also by the clinical characteristics of the children participating in the study.

Despite the great innovativeness of the present study in shedding new light on the pathways associated with BIF, our study is not free from some limitations. In particular, the number of diffusion directions in the DWI sequence might appear as limited when compared to the state-of-the-art MRI acquisitions. However, considering the particular cohort of subjects included, more prone to movement during the examination (14 DWI datasets were discarded for elevated head motion), the chosen DTI sequence represents a good trade-off between having qualitatively good data and acquisition time. Another limitation is represented by the above-mentioned link between the intellectual functioning and the neuropsychiatric conditions that prevents the possibility to clearly distinguish between the two factors in the interpretation of the results. However, this limitation is hardly avoidable when investigating children exposed to ELA presenting with BIF. Finally, characterizing brain network is particularly challenging especially when applied to brain development because of the dramatic changes occurring. In this regard, the small number of healthy controls included represents a limitation to the power of the study. Nevertheless, the comprehension of brain maturation mechanism and the neural plasticity is warranted by the accurate and relevant normalizations of MRI data that allow for comparisons between groups, especially regarding neurodevelopmental disorder

for which gross brain abnormalities are lacking (50) as in the case of BIF.

Taken together all these data indicate that the neurobiological underpinning of the clinical manifestations of children exposed to ELA associated with BIF is represented by the reduced GE in information integration and by the altered structural connectivity in the circuitry crucial for the regulation of emotions, behavior, motivation, and memory. These abnormalities are closely related to the IQ of children. We consider these data extremely relevant for the understanding of the cognitive and behavioral manifestations of these children and for the implementation of appropriate rehabilitative interventions able to reduce the risk of future psychiatric disorders in these children.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available because: The informed consent approved by the Ethics committee did not include any statement regarding the possibility to share the data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committees of the Don Gnocchi Foundation and of the ASST S. Paolo and S. Carlo Hospital. All parents signed a written informed consent at the first meeting. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. VB, MCle, and FB designed and supervised the research, interpreted the results, and drafted the manuscript. AP and MCab performed MRI data analysis and drafted the manuscript. ST and ML performed the statistical analysis. AG, GB, MZ, MCan, and MW recruited patients. Furthermore, AG and GB performed the behavioral and neuropsychological evaluations. MZ also contributed in the analysis of the clinical data.

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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.2020.497116/full#supplementary-material

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BRIEF RESEARCH REPORT
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Psychiatric Symptomatology, Mood Regulation, and Resting State Functional Connectivity of the Amygdala: Preliminary Findings in Youth With Mood Disorders and Childhood Trauma

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Background: As mood dysregulation and hyperarousal are overlapping and prominent features of posttraumatic stress disorder (PTSD), and mood disorders (MD) including bipolar disorder (BD), we aimed to clarify the role of trauma and MD on the resting state functional connectivity (RSFC) of amygdala in MD youth with or without trauma exposure, and healthy controls (HC).

Methods: Of 23 subjects, 21 completed the magnetic resonance imaging (MRI) protocol, 5 were excluded for subject motion, leaving final sample size of 16: nine subjects with MD (5/9 with trauma), and 7 HC. Youth were assessed with Schedule for Affective Disorders and Schizophrenia for School Aged Children—Present and Lifetime Version (K-SADS-PL), and other behavioral measures including Young Mania Rating Scale (YMRS). Imaging data were acquired using functional MRI in 3-T scanner. Imaging included T1-weighted structural MRI and 6-min resting state acquisition.

Results: In between group analysis, the average correlation coefficients between left anterior cingulate cortex (Acc) and left insula cortex with left amygdala regions were significantly larger in HC compared to the patient population. Connectivity between left amygdala and left cingulate cortex shows a significant negative correlation with YMRS severity.

Conclusions: In this preliminary study, MD with trauma youth had more manic symptoms and difficulties regulating anger. While MD youth showed reduced RSFC of left amygdala with left acc and left insula, no significant difference between the subgroups of children with MD was observed. However, when looking at both clinical groups together, we observed a significant correlation of RSFC of left amygdala to left acc, and YMRS scores.

Keywords: mood regulation, resting state functional connectivity, amygdala, trauma, magnetic resonance imaging

INTRODUCTION

Identifying the contributions of childhood trauma to the development and presentation of mood disorders (MD) is an important task for clinicians working with affected youth (1), especially given known contributions of childhood trauma to mood dysregulation and more severe presentations of MD (2). Assessing for biomarkers of illness by using neuroimaging is a powerful way to address this diagnostic quandary. Given that deficits in emotion processing and hyperarousal symptoms are overlapping and prominent features of posttraumatic stress disorder (PTSD), and MD including bipolar disorder (BD), studies are needed in order to highlight disorder-unique versus common psychopathologies. We sought to clarify the role of trauma and MD on the resting state functional connectivity (RSFC) of the amygdala in youth with MD with or without trauma exposure as well as healthy controls (HC).

Understanding the association between trauma and the development of MD will increase our knowledge of the diverse effects of such events on youths' emotional and behavioral development. Specifically, it is important to look at mood dysregulation as a central symptom across diagnostic groups, as mood regulation/dysregulation may have important implications in terms of treatment approaches, biological markers, and social/demographic factors. In this context, we are specifically interested in investigating the brain function of the amygdala, a brain region which mediates aspects of social-emotional functioning, and participates in processing information about significant emotional stimuli.

Amygdala and RSFC in Youth With MD

Relative to HC, several RSFC studies have shown that youth diagnosed with major depressive disorder (MDD) exhibit hypoconnectivity between the amygdala and the dorsolateral prefrontal cortex and the anterior insula (3). Bebko and colleagues (4) showed a trend toward an inverse relationship between the RSFC between the amygdala-bilateral posterior insula and the parent general behavior inventory item scale, demonstrating that youth who exhibited increasingly dysregulated behaviors had lower functional connectivity between the amygdala-bilateral insula (4). Greater positive connectivity between the ipsilateral amygdala and insula has been associated with greater remission in depressive symptoms over time (5). When comparing the functional connectivity of patients with MDD who completed cognitive behavioral therapy (CBT) treatment, these individuals display increased connectivity of their amygdala to their insula, anterior cingulate cortex (Acc), and dorsolateral prefrontal cortex relative to their pre-CBT connectivities (3). Finally, in a study by Pannekoek and colleagues (6), the left amygdala demonstrated hyperconnectivity to the inferior frontal gyrus in individuals with depression (6).

Amygdala Connectivity in Traumatized Adolescents

Thomason and colleagues (7) showed that there was a decrease in negative connectivity between the insula and the amygdala in urban youth who had experienced childhood maltreatment, suggesting reduced emotion regulation control/loss of inhibitory affective control in those youth (7). Marusak and colleagues (8) demonstrated that trauma exposed youth had increased connectivity within the amygdala and insula relative to youth not exposed to trauma (8). Reduction in amygdala-insula connectivity during tasks that require cognitive reappraisal have been correlated with greater symptom reduction in adolescent girls treated with Trauma Focused CBT. However, girls that continued to exhibit increased amygdala-insula connectivity during tasks that require cognitive reappraisal had less abatement of symptoms associated with PTSD. The authors hypothesize that this change indicates decreased interoceptive representation of negative affective states (9).

Adolescents with PTSD have been shown to exhibit hypo-connectivity between amygdala and frontal structures including the dorsolateral and ventromedial cortex, Acc, and hippocampus. Herringa and colleagues (10) found Childhood Trauma Questionnaire (CTQ) scores to be inversely correlated with the RSFC of right amygdala and subgenual Acc. This study also showed that reduced amygdala-hippocampal connectivity was associated with increased internalizing symptoms (10).

Amygdala Connectivity in MD and Trauma

Few studies have researched RSFC in co-occurring PTSD and MD in adolescents. Sun and colleagues (11) examined the effects of early life abuse as measured by CTQ in adolescents with depression who were overweight. The study demonstrated decreased connection between the amygdala and precuneus, with less negative connectivity in adolescents with depression who were overweight and experienced high levels of abuse relative to adolescents with depression who were overweight and experienced low levels of abuse. Those with depression who were overweight and experienced high levels of abuse exhibited decreased connectivity, and less negative interaction between the insula and the precuneus relative to their low-level abuse counterparts (11).

In adults with co-occurring PTSD and MDD, Kennis and colleagues (12) demonstrated increased RSFC between the hippocampus and the insula relative to subjects with PTSD alone. Of note, the difference between the two groups became insignificant when subjects taking psychotropics were excluded from this analysis. This study also showed increased connectivity between the subgenual Acc and the perigenual Acc in the PTSD +MDD group. There was a negative correlation between the connectivity of these two structures and re-experiencing symptoms of PTSD (12). Zhu and colleagues (13) also sought to study differences in RSFC in adults with co-occurring PTSD and MDD, PTSD alone, and trauma-exposed healthy individuals. In this study, individuals with PTSD+MDD versus PTSD alone exhibited significantly decreased RSFC in basolateral amygdala and orbitofrontal cortex. This connectivity inversely correlated to severity of MDD symptoms in all three subject groups (13). Satterthwaite and colleagues (14) examined the relationship between depressive symptoms in women with the diagnosis of MDD, PTSD, and HC and resting connectivity and showed depression severity to be linked to decreased RSFC

between amygdala and anterior insula, dorsolateral prefrontal cortex, and Acc (14).

Hypothesis

Given this literature and our prior expectations, we hypothesize that children with MD trauma histories will exhibit abnormal connectivity between amygdala and frontal lobe, when compared to children with MD without trauma and HC.

APPROACH

Subjects

We studied three samples consisting of 8- to 12 year-old children: 1) MDT: Children who have experienced trauma (parent reported history of significant interpersonal trauma between the ages of 0-5 years of age) and have MD (Mood Disorder Not Otherwise Specified, MDD or BD, with Clinical Global Impression Scale (CGI-S) score ≥ 3 and a Young Mania Rating Scale (YMRS) score ≥ 8) (N=5); 2) MD: Children who have MD without trauma (N=4); and 3) HC: Healthy Controls without trauma experience or mood symptoms (N=7). Exclusion criteria included a history of head trauma, current serious suicide risk, and co-occurring current psychosis, substance use, ASD and ID, as well as contraindications for MRI. HC were excluded if they had a psychiatric diagnosis or a first degree relative with BD, MDD, or schizophrenia. Enrollment and consent procedures for this study were approved by the institutional review board at our institution.

Clinical and Behavioral Assessments

The following evaluations were completed by a trained child psychiatrist or child psychologist. All youth received a diagnostic assessment using the Schedule for Affective Disorders and Schizophrenia for School Aged Children—Present and Lifetime Version (K-SADS-PL) (15) to identify MD and other co-occurring psychiatric diagnoses. A supplementary module was used to assess the severity of mood dysregulation (abnormally angry or sad mood, over-reactivity to negative emotional stimuli). In addition, subjects were assessed for general psychiatric symptomatology using the Brief Psychiatric Rating Scale for Children (BPRS-C) (16), and for mood symptoms using the Young Mania Rating Scale (YMRS) (15), and Children's Depression Rating Scale-Revised (CDRS-R) (17). Mood regulation was additionally assessed using the Children's Emotion Management Scales: Anger and Sadness (CSMS&CAMS) (parent and child reports) (18), and Emotion Regulation Checklist (19). Subjects were assessed for trauma exposure and PTSD symptomatology using the CTQ (20), and UCLA PTSD index for DSM IV (Child and Parent report) (21). Executive function was assessed using the Behavior Rating Inventory of Executive Function (BRIEF) (22). Other clinical information obtained during the psychiatric clinical assessment included: demographic characteristics and socioeconomic status, number of medications and types, the percent of individuals with a lifelong history of psychiatric hospitalization/out of home placement, family history of psychiatric illness and substance use disorders, MRI safety screening questionnaire, and head circumference, height, and weight of subjects.

Image Data Acquisition

Imaging data were acquired using a 3 Tesla Philips Achieva whole-body MR system (Philips Healthcare, Best, The Netherlands) with an eight-element phased-array head coil. Imaging included a T1-weighted structural MRI (MPRAGE sequence, 256×256 voxels; TR: 6.985 ms; TE: 3.15 ms; FOV: 240 mm×256 mm×180 mm; 180 slices), and a 6-min resting state acquisition (TR 2s; TE 35ms; Flip Angle 80°; image matrix 128x128; resolution; FOV 230x230mm; slice thickness 3mm; 35 axial slices).

Data Analysis

Resting state analyses were performed using the Functional Connectivity (CONN) toolbox version 17.f (23) using routines from the Statistical Parametric Mapping software (SPM12; RRID : SCR_007037, Wellcome Trust Centre for Neuroimaging, London, UK) using MATLAB 2016b. Image preprocessing included: realignment to correct for motion, slice timing correction, and spatial transformation to standard MNI space prior to statistical analysis. The participants with movement where scrubbing flagged more than 60 time-points (⅓ of the run) across the run were rejected. Effects of nuisance variables (global, white matter and CSF signals, and movement parameters) were included in the denoising step; finally, data was band-pass filtered to 0.008–0.09 Hz.

Temporal correlations of the resting-state BOLD signal time series were examined between the left amygdala "seed" region [anatomically derived regions of interest from the automated anatomical labeling (AAL) toolbox] and the rest of the brain (seed-to-map correlation map). Based upon our hypotheses, we focused our attention on ipsi-lateral (left hemisphere) in the frontal lobe. For the group-level statistics, we used 0.001 as the cluster-forming threshold and a FWE threshold of p=0.05 (two-tailed). We modeled between group differences (CTL vs. PTS) in these maps (controlling for mean motion, age, and gender). For the correlation of left amygdala RSFC with symptom severity, we also controlled for mean motion, age, and gender.

Statistical analyses were performed using the R Project for Statistical Computing (24). Clinical and behavioral assessments were compared among the groups using ANOVA for continuous measures with post-hoc paired comparisons based on the Tukey Honestly Significant Difference method for constructing confidence intervals for the observed mean difference. The presence of co-morbid diagnoses among the groups were evaluated by Chi-square goodness of fit. Demographic and physical data are compared using similar methods for continuous and categorical measures with no correction for multiplicity.

RESULTS

Of the 23 subjects, 21 completed the MRI protocol. Of these, 5 were excluded due to subject motion issues, leaving the final sample size of 16 (MDT; N=5, MD: N=4, HC; N=7). Tables providing the

demographic characteristics of this final imaging subgroup (**Supplementary Table 1**) and their co-occurring diagnosis (**supplementary table 2**) are available as supplementary materials. Eight of the nine subjects in the two clinical groups reported taking psychotropic medications (compared to none in the HC group), and all but two reported taking three or more medications.

Behavioral Measures

The group-wise behavioral measures are reported in **Table 1**.

The BPRS measures general psychiatric symptomatology. Subjects in the MDT group scored the highest on this scale (mean: 60.8, 95% CI: 49.8, 71.7), followed by MD group (mean: 47.5, 95% CI: 23.0, 72.0) and HC (mean: 24.1, 95% CI: 21.8, 26.5). A similar pattern is seen when looking at the behavioral subscale of the BPRS (MDT=15.8 (11.0, 20.6); MD=8.8 (0, 20.2); HC=3.4 (2.9, 3.9)) and the mania subscale of the BPRS (MDT=15.8 (10.9, 20.7); MD=10.7 (0, 21.8); HC=3.4 (2.4, 4.5)). The depression subscale of the BPRS suggested no difference in report of depressive symptoms between the two clinical groups; however, both clinical groups reported significantly more depressive symptoms than HC.

Mood Symptoms

The three groups significantly differed in report of manic symptoms as measured by YMRS. Youth in the MDT group had the highest scores (mean=19.8, 95% CI: 13.0, 26.6), followed by youth in the MD group (mean=9.5, 0.27, 18.7) and HC (mean=0.1, 0, 0.5). There was no difference in report of depressive symptoms as measured by the CDRS between the two clinical groups; however, the MDT group reported significantly more depressive symptoms than HC (mean difference=14.6 (95% CI: 4.3, 24.9), p=0.007).

Emotional Regulation

Parents reported on their children's ability to regulate emotions using the ERC. Subjects in the two clinical groups scored significantly higher on ERC subscale 1 assessing lability/negativity (higher scores suggest greater dysregulation) and significantly lower on ERC subscale 2 assessing emotional regulation, compared to HC (ERC 1: mean difference (MDT-HC)=23.8 (95% CI: 13.4, 34.1), p= 0.0001; mean difference (MD-HC)=16.5 (95% CI: 5.4, 27.6), p=0.004; ERC2: mean difference (MDT-HC)= -7.5 (95% CI: -11.6, -3.3), p= 0.0011; mean difference (MD-HC) = -9.4 (95% CI: -13.8, -4.9), p=0.0003).

Using the CEMS—parent version, and the CEMS—child version, parents and youth were asked to rate the children's responses to emotions of sadness, anger, and worry. On the CEMS anger subscale, parents reported that MDT youth had significantly lower coping skills in regards to anger compared to MD youth (mean difference = -5.8 (95% CI: -9.8, -1.8), p=0.006) and slightly lower than HC (mean difference= -3.4 (95% CI: -7.2, 0.4), p=0.08). Parents of MDT youth also reported that their child has significantly lower coping skills in regards to worry compared to MD (mean difference = -4.3 (95% CI: -7.5, -1.1), p=0.01) and HC (mean difference = -3.8 (95% CI: -7.0, -0.6), p=0.021).

TABLE 1 | Behavioral Measures.

Rating Scale	MDT Mean ± SD	MD Mean ± SD	HC Mean ± SD	Comparisons *
Clinician Administered				
General Symptoms				
BPRS Total	60.8 ± 8.8	47.5 ± 15.4	24.1 ± 2.5	MDT & MD > HC
BPRS Behavioral	15.8 ± 3.9	8.8 ± 7.2	3.4 ± 0.5	MDT > HC
Mood Symptoms				
BPRS Mania	15.8 ± 4.0	10.8 ± 6.9	3.4 ± 1.1	MDT & MD > HC
BPRS Depression	9.4 ± 5.3	8.8 ± 5.9	3.7 ± 1.0	
YMRS	19.8 ± 5.5	9.5 ± 5.8	0.1 ± 0.4	MDT > MD > HC
CDRS	32.6 ± 11.3	24.8 ± 3.8	18.0 ± 2.2	MDT > HC
Mood Regulation				
ERC Lability/Negativity	43.8 ± 5.6	36.5 ± 11.6	20.0 ± 3.1	MDT & MD > HC
ERC Emotion Regulation	23.4 ± 3.6	21.5 ± 3.1	30.9 ± 1.5	MDT & MD > HC
CEMS Sadness Parent	19.8 ± 2.0	23.5 ± 3.7	22.8 ± 3.6	
CEMS Anger Parent	16.2 ± 1.6	22.0 ± 2.2	19.6 ± 2.7	MDT < MD
CEMS Worry Parent	14.2 ± 1.9	18.5 ± 0.6	18.0 ± 2.2	MDT < MD & HC
CEMS Sadness Child	24.4 ± 2.6	19.0 ± 7.0	24.6 ± 1.4	
CEMS Anger Child	21.4 ± 1.7	21.3 ± 2.6	22.4 ± 2.0	
CEMS Worry Child	18.8 ± 3.3	17.5 ± 3.9	19.9 ± 1.3	
Trauma and PTSD Symptoms				
UCLA PTSD Parent	20.4 ± 20.1	0 ± 0	0 ± 0	MDT > MD & HC
UCLA PTSD Child	5.0 ± 10.6	0.3 ± 0.5	1.1 ± 3.0	
CTQ Total	32.5 ± 5.6	30.3 ± 4.1	26.7 ± 2.8	
Executive Function				
BRIEF Global Executive Composite (T score)	73.4 ± 11.1	74.5 ± 19.4	42.7 ± 5.5	MDT & MD > HC
BRIEF Behavioral Regulation Index (T score)	67.0 ± 13.4	70.8 ± 7.2	42.2 ± 4.4	MDT & MD > HC
BRIEF Metacognition Index (T score)	70.6 ± 12.8	74.0 ±	42.0 ± 2.8	MDT & MD > HC

** Significant pairwise differences between means adjusted for familywise error based on Tukey Honestly Significant Difference.*

There were no significant differences between the three groups in terms of total scores on the child ratings of anger, worry, or sadness, in the ability to inhibit sadness, worry, and anger, or in the ability to regulate the feeling of sadness and worry.

Trauma and PTSD Symptoms

CTQ total score was on average 5.8 points higher in the MDT group than for HC, a difference which is modestly significant when correcting for multiple comparisons (95% CI: -0.8, 12.4; p=0.09). There were no significant differences in the UCLA PTSD child version amongst the three groups. In the UCLA PTSD parent version MDT youth were reported to have higher PTSD symptoms compared to MD youth (mean difference = 20.4 (95% CI: 0.7, 40.1), p=0.04), and HC (mean difference=20.4 (95% CI: 3.2, 37.6), p=0.02). The MD and HC groups both reported no PTSD symptoms on this scale.

Executive Function

Parents of youth in both clinical groups rated their children significantly higher on all categories of the BRIEF relative to parents of HC (see **Table 1**; BRIEF Global Executive Composite T-score: mean difference (MDT-HC)=28.6 (95% CI: 13.1, 44.1), p=0.0009; mean difference (MD-HC) = 32.0 (95% CI: 15.5, 48.5), p=0.0006), with the other composite scales having a similar profile).

IMAGING RESULTS

Figure 1 shows the between group (Patients versus Controls) differences in the left hemisphere of the connectivity of all voxel time courses with the mean left amygdala time course. Specifically, this is presented as an inflated hemispheric surface map of the medial surface of the left hemisphere, where regions that are significantly different between patients and controls are colored. There are two spatially contiguous regions identified, both in the frontal lobe. The first cluster, virtually completely contained within the left Acc region of the CONN atlas (coordinate (-4, +22, +28)), is comprised of 135 voxels and has a family-wise error (FWE) significance of 0.000284. The second cluster, residing mostly in left insula cortex (coordinate (-40, +04, -12)), is comprised of 67 voxels with FWE significance of 0.027330.

The individual average correlation values of voxels in these clusters with the left amygdala seed reference time course were extracted for each subject. **Figures 1C, D** show the box plot for the patient and control subjects for the left Acc and Insula

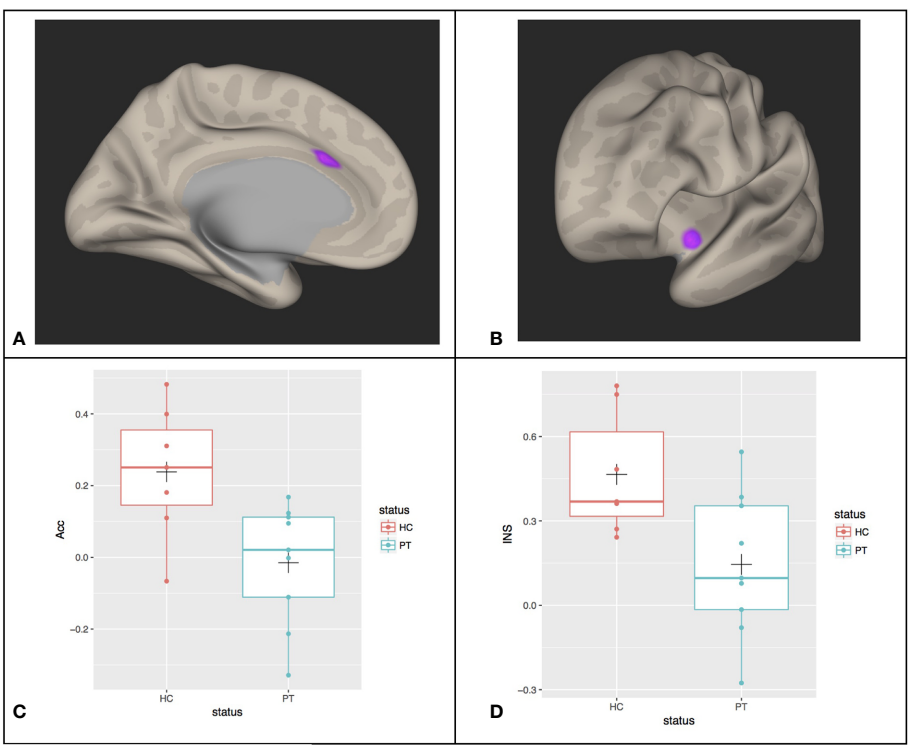

FIGURE 1 | Between group differences (PT, patients; HC, healthy controls) in the functional connectivity of all voxel time courses with the mean Left Amygdala time courses in **(A)** left anterior cingulate cortex (Acc) and **(B)** left insular cortex (INS). The mean connectivity values in these regions of significance were extracted for each participant and shown in **(C, D)** with boxplots overlayed. The cross ("+") indicates the location of the group mean.

regions, respectively. In the left Acc region, HC subjects have an average correlation coefficient between this cluster and the left Amygdala of approximately 0.24 (SD=0.18), whereas the patients' correlation is reduced to approximately -0.015 (SD=0.17) (this difference is significant: T(14)= -2.9, p=0.013). For the insula cortex cluster, controls show approximately 0.47 (SD=0.22) correlation with the left amygdala, which is reduced to 0.15 (SD=0.26) in the patients (T(14)= -2.6, P=0.02).

We examined if, within the patient population, there is a difference between MDT subjects and MD subjects in this resting-state correlation with left amygdala. These data are shown in **Figure 2**, with the HC subjects for comparison. No significant differences in connectivity due to trauma exposure was identified (mean difference (MDT-MD) = -0.11 (95% CI: -0.42, 0.20), p = 0.6).

Correlation With Mood Symptoms Severity

Correlation of connectivity and CDRS in all subjects, controlling for (all subjects, age, gender, mean motion) was calculated. No significant regions were observed at the specified thresholds. We did observe a subthreshold region in the left fronto-orbital cortex (compromised of 171 voxels with a cluster forming threshold of 0.005).

Correlation of connectivity and YMRS in all subjects, controlling for (all subjects, age, gender, mean motion) is shown in **Figure 3**. Two regions are identified, but only one is in the frontal lobe. This frontal region lies within the left hemisphere Acc and paracingulate cortex. For this region, the FWE was 0.000078. Within this cluster, we plot the individual correlation value with the left amygdala seed region as a function of YMRS score. This yields a significant negative correlation (r (14) = -0.85, p = 0.0000279) of this regional connectivity with the behavioral scale: connectivity between the left amygdala and left cingulate cortex decreases as YMRS severity increases.

DISCUSSION

Behavioral measures suggest that MDT youth had more severe psychiatric presentations as measured by the BPRS, and more significant manic symptoms as measured by the BPRS mania subscale and the YMRS. In addition, this group had more significant difficulties regulating anger, as measured by the CEMS parent report. Interestingly, CTQ scores were only modestly elevated in the youth who have experienced trauma. This is likely because this group experienced trauma before age 5

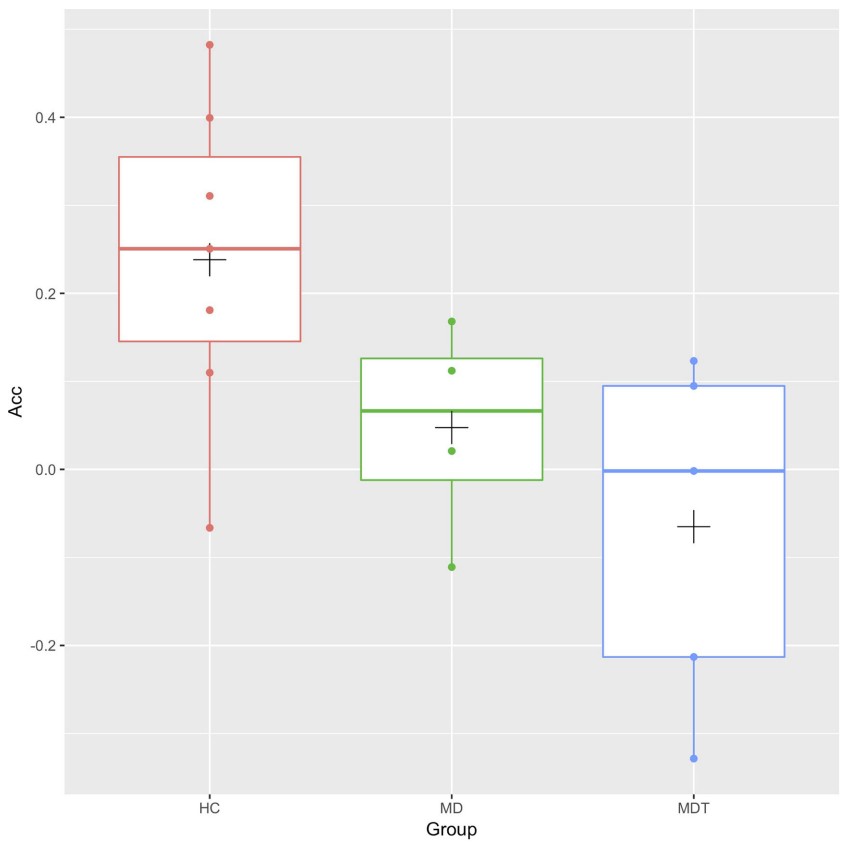

FIGURE 2 | Box plots of the resting-state functional connectivity between the left Anterior Cingulate Cortex (Acc) and the left Amygdala for MDT (Mood Disorders and Trauma), MD (Mood Disorders), and HC (healthy controls) subjects. The cross ("+") indicates the location of the group mean.

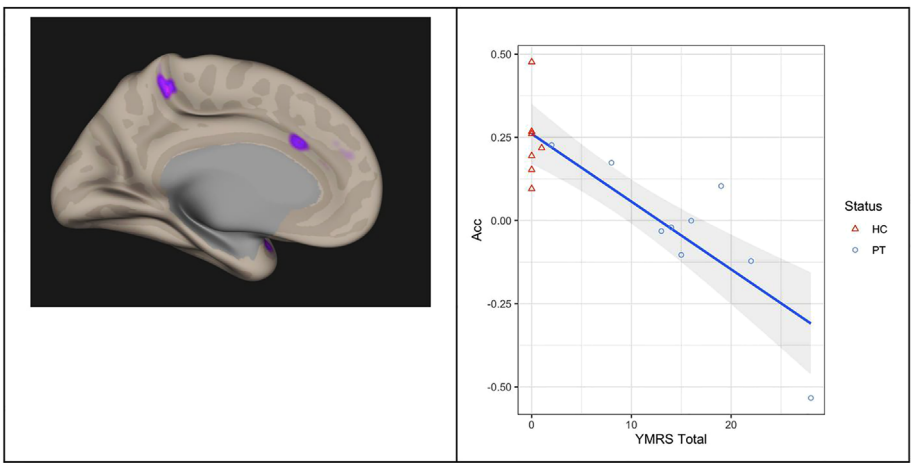

FIGURE 3 | Correlation of functional connectivity and YMRS Total score in all subjects: connectivity between the left Amygdala and left Anterior Cingulate Cortex (left panel) decreases as YMRS severity increases (right panel). The line of linear best fit is shown with 95% confidence bands, with the groups distinguished by color for visualization only. Acc, anterior cingulate cortex; YMRS, young mania rating scale; PT, patients; HC, healthy controls.

years, and therefore did not have verbal memory of the events to report. In addition, only parents reported significant PTSD symptoms in the MDT group on the UCLA PTSD.

With RSFC study, we sought to explore the hypothesis that children with MD and trauma histories will exhibit abnormal connectivity between the amygdala and frontal lobe, when compared to children with MD without trauma and HC. Overall, while children with MD did show reduced RSFC of left Amygdala with left Acc and left Insula, we did not observe a significant difference between the subgroups of children with MD with and without trauma histories. However, when looking at both clinical groups (MT and MDT youth) together, we observe a significant correlation of RSFC of left Amygdala to left Acc, and YMRS scores. To the extent that a history of trauma may be related to increased severity of MD, the trauma factor may be exacerbating the functional connectivity alterations between these regions.

With respect to our specific hypothesis (that children with MDT will exhibit abnormal connectivity between amygdala and frontal lobe, when compared to children with MD without trauma and HC), we did not observe a significant trauma-history-specific change in RSFC separate from the diagnosis-specific changes we observed. However, the relationship between disorder severity (which is associated with history of trauma separately) and degree of RSFC change indicated the potential for an interaction that we didn't have the power to resolve.

Anatomic regions featured in our findings included left amygdala, left insula and left Acc. The **salience network** is a 'circuit' that includes these regions, consisting of the two key nodes, the **anterior insula** and the **Acc**. It is also comprised of subcortical regions including the **amygdala**, dorsomedial thalamus, hypothalamus, ventral striatum, and the substantia nigra/ventral tegmental area (25). This system is integral in the top down appraisal of novel stimuli. The **insula** is involved in processing of emotions and indicated in switching between different networks, including the default mode network and central executive network. By doing so, it is thought that the insula serves to modulate behavioral responses to salient stimuli (26). Much of the salience network is comprised of structures that are also part of the limbic system; the **amygdala**, which is part of the salience network and the limbic system, is involved in the appraisal of emotionally salient stimuli from our environment and integration of these stimuli with previously processed data (27). Our findings of connectivity alterations between amygdala and Acc and insula are similar to a number of prior studies. Specifically, our findings are in line with those of Bebko and colleagues (dysregulated behaviors correlated with lower functional connectivity between the amygdala-bilateral insula) (4) and Straub and colleagues (hypoconnectivity between amygdala and dorsolateral prefrontal cortex and anterior insula in MDD) (3), in that we see altered RSFC between the amygdala and insula, as a potential bio-marker of behavioral/emotional symptoms (and disorder severity), manifesting as decreased ability to regulate emotions.

We acknowledge a number of limitations to this study. First, the sample size (final sample size of 16 (MDT; N=5, MD: N=4, HC; N=7) is admittedly small. As such, these findings and interpretations should be considered as preliminary, pending larger replication studies which can build further upon these initial observations. Second, we took a very conservative single seed-based approach to examination of RSFC (left amygdala connectivity to left frontal regions) which focused (and also 'limited') the scope of types of changes we would observe. While this helps reduce the number of comparisons made, it can also bias our interpretations to specific networks.

CONCLUSIONS

In conclusion, we believe that our pilot imaging study is unique due to the population of youth studied: those with MD and

histories of childhood trauma. Although our study was focused on finding a significant trauma-history-specific change in RSFC (which we did not identify), we did find that mechanisms of dysconnectivity were associated with symptom severity across clinical groups (MDT and MD). Given that there were differences in mania and coping with anger in MDT group, these seem to be clinical markers for the additional illness burden in traumatized youth with a signal in the imaging data. Clinically, screening for mania in traumatized youth, treating anger coping skills, and screening for trauma in youth with MD appear to be important conclusions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UMass Medical School Human Subjects Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YD was the primary investigator designing the study and lead author. DK lead imaging effort. SH conducted all statistical analysis of behavioral measures and imaging. DP contributed significantly to researching background materials and writing the manuscript. BD assisted with design of behavioral part of the study and conducted clinical interviews. JF was the senior mentor on the project providing guidance to design of study and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.525064/full#supplementary-material

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REVIEW
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Premature Birth and Developmental Programming: Mechanisms of Resilience and Vulnerability

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The third trimester of pregnancy represents a sensitive phase for infant brain plasticity when a series of fast-developing cellular events (synaptogenesis, neuronal migration, and myelination) regulates the development of neural circuits. Throughout this dynamic period of growth and development, the human brain is susceptible to stress. Preterm infants are born with an immature brain and are, while admitted to the neonatal intensive care unit, precociously exposed to stressful procedures. Postnatal stress may contribute to altered programming of the brain, including key systems such as the hypothalamic–pituitary–adrenal axis and the autonomic nervous system. These neurobiological systems are promising markers for the etiology of several affective and social psychopathologies. As preterm birth interferes with early development of stress-regulatory systems, early interventions might strengthen resilience factors and might help reduce the detrimental effects of chronic stress exposure. Here we will review the impact of stress following premature birth on the programming of neurobiological systems and discuss possible stress-related neural circuits and pathways involved in resilience and vulnerability. Finally, we discuss opportunities for early intervention and future studies.

Keywords: prematurity, stress, hypothalamus-pituitary-adrenal axis, autonomic nervous system, large-scale brain networks, epigenetics, resilience

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INTRODUCTION

The third trimester of pregnancy represents a sensitive phase for infant brain plasticity, as a series of fast-developing cellular events, such as synaptogenesis, neuronal migration, and myelination regulate the development of neural circuits (1). Throughout this period of growth and development, the human brain is highly susceptible to stress exposure. Very preterm infants are born with a neurobiological immature system and are precociously exposed to stressful procedures during weeks to months in the Neonatal Intensive Care Unit (NICU). The excessive and prolonged exposure to stress during NICU admission can exceed the infant's natural regulatory capacity, threatening the allostatic balance of the infant, and might permanently alter neuroendocrine, autonomic, cardiovascular, and neural responses (2), leading to persisting mental morbidity throughout the lifespan (3, 4).

Along with increased survival in extremely preterm born infants (EP; gestational age <28 weeks) due to continued progress in perinatal care (5, 6), the rates of dysfunction in the area of mental health and behavior have remained unchanged or even worsened during the last decades

(7–10). With an increased risk for a wide spectrum of psychiatric disorders, the preterm phenotype is primarily represented by deficits in attention, executive functioning, and emotional symptoms. Importantly, whilst preterm birth is associated with a higher prevalence of psychiatric disorders, a large proportion of children remain relatively unaffected [e.g., (11)].

Here we will review the impact of stress following prematurity on the programming of neurobiological systems. We begin by giving a short overview of the different types of stressors observed in postnatal stress research, followed by the typical development of autonomic, endocrine, and top-down regulatory systems in the fetal period. We then turn to evidence that postnatal stress following prematurity has short- and long-term effects on brain development. Lastly, we discuss possible mechanisms by which postnatal adversity increases the risk for social and affective problems following prematurity. Throughout this review, we call attention to critical gaps and unanswered questions and make suggestions for future research elucidating the mechanisms linking postnatal stress, neurobiology, and future social and affective development. Where appropriate, we focus on providing evidence from human postnatal studies; however, we rely on prenatal and/or animal studies where investigations of critical questions in preterm individuals are lacking.

SOURCES OF STRESS AFTER PREMATURE BIRTH

In the current review, we define stressors as any "*real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses*" [(4), p. 508]. In full recognition of the fact that there are numerous categorizations and types of stressors, including the administration of synthetic corticosteroids (e.g., dexamethasone) and its detrimental impact on postnatal development [e.g., (12)], for the purpose of this review we divided postnatal stressors into physical, environmental, and maternal stimuli or events. It is important to note that the effects of neonatal stress might be confounded by prenatal factors causative of preterm birth, such as vascular disease and infections (13), and hence both mechanisms may coexist. Also, the abrupt loss of intrauterine neurotrophic support following preterm birth could have deleterious effects on developmental programming, with reported damage to oligodendrocytes in the developing nervous system [for a review see (14)]. These prenatal factors are both highly relevant but are beyond the scope of the current review.

Physical Stress

Physical stress of repetitive procedural pain occurs routinely in extremely preterm neonates who are admitted to the intensive care unit. Preterm infants and neonates show an increased physiological and behavioral sensitivity toward painful procedures, as their pain transmission and modulation are still underdeveloped [for a review see (15)]. Due to a disbalance between afferent excitatory neurotransmitters and the descending inhibitory neurotransmitters, this hypersensitivity to pain is exacerbated in preterm infants (16, 17). There are currently two main categorizations of pain-related stressors: (1) acute procedural, (2) and acute prolonged (18). Acute procedural stress is triggered by a specific noxious stimulus (19), such as a heel stick, whereas acute prolonged stress represents a longer time duration with a distinguishable beginning and expected endpoint, such as mechanical ventilation or surgery (20).

Although we currently only defined physical neonatal stress as painful procedures and interventions; medical complications, such as hypoxia, infections, and inflammation, could also be considered as extremely stressful for preterm-born individuals. Hence, the current description of physical stress is not exhaustive.

Environmental Postnatal Stress

The effects of the nursery environment on the preterm infant, such as sound levels and nursing interventions, have become an area of concern for research. Studies showed that noise levels often exceed the American Academy of Pediatrics-recommendation of 40–45-dB (21), with NICU sounds ranging from 50 to 90 dB (22). Continuous loud noise has deleterious physiological effects on preterm infants and induces stress behaviors. More specifically, excess auditory stimulations have been associated with decreased oxygen saturation, increased heart rate and blood pressure, and alterations in sleep-wake state [for a review see (23)]. To reduce these unfavorable environmental factors, recent effort has been put into investigating the effects of single-family rooms vs. open bay units [for a review see (24) and section Opportunities for Early Intervention].

Physicians and nurses have rated routine caregiving events in the NICU as stressful to preterm infants (25). Indeed, common nursing interventions, such as diaper changes, noise, and light, can elicit similar stress-like responses as with invasive procedures [e.g., salivary cortisol levels, crying, heart rate (26, 27)]. However, to date, the impact of caregiving-related stress on the developing brain is not thoroughly researched.

Maternal Care

Preterm infants generally experience atypical maternal care while admitted to the NICU, whilst maternal behavior toward their preterm infant plays a crucial role in the early regulation of the infant's stress responses (28). Both physical and emotional closeness might become obstructed, subsequently increasing feelings of separation and negatively impacting mother-infant attachment (29, 30). Interestingly, previous studies found that both mother's mental well-being and the physical conditions of the infant contribute independently to the degree of maternal attachment (31). More specifically, preterm infants may be regarded as less "rewarding social partners," as their neurological immaturity negatively impacts their social responsiveness [e.g., less time in alert state (32–34)]. In other words, preterm infants might be less responsive to parental cues and show more negative expressions, which might negatively impact the quality of mother-infant interaction [for a review see (35)]. In turn, due to the circumstances, mothers of preterm infants tend to spend less time holding, talking to, and looking at their premature infant compared to mothers of full terms (36).

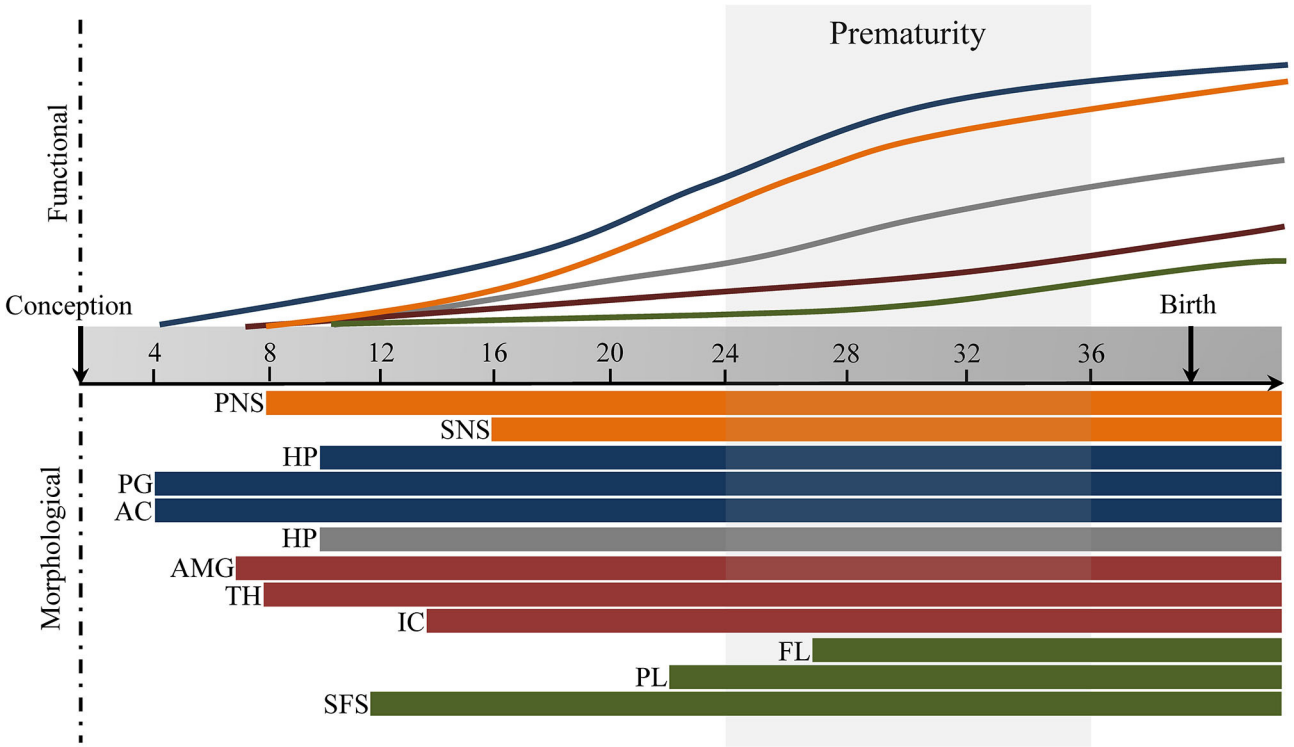

FIGURE 1 | Fetal development of stress-response and stress-regulatory brain networks for illustration purposes. Autonomic Nervous System (orange), Hypothalamic-Pituitary-Adrenal axis (blue), Salience Network (red), Executive Control Network (green). PNS, parasympathetic nervous system, SNS, sympathetic nervous system, HPT, hypothalamus, PG, pituitary gland; AC, adrenal gland; HP, hippocampus; AMG, amygdala; TH, thalamus; IC, insular cortex; FL, frontal lobe; PL, parietal lobe; SFS, superior frontal sulcus. Timing based on literature reviewed under section Pre- and Post-natal Brain Development of Stress Systems and Brain Networks. Default Mode Network (gray).

PRE- AND POST-NATAL BRAIN DEVELOPMENT OF STRESS SYSTEMS AND BRAIN NETWORKS

Fetal brain development is characterized by large maturational changes in volume, as well as changes in microstructural and functional connectivity, which has broad implications for future development (37). These maturational changes are regulated by complex molecular and cellular processes, such as neurogenesis and neuronal migration, synaptogenesis, and axonal growth [for an overview of the spatial progression, the reader is referred to (38)]. The endocrine and autonomic stress-systems are fundamentally shaped during the fetal period. Here we will give a short overview of the spatiotemporal changes involved in the development of the autonomic nervous system (ANS), hypothalamic-pituitary-adrenal axis (HPA-axis), and stress-related brain networks, and their role in the fetal stress response (see **Figure 1**).

Fetal Development of the Autonomic Nervous System (ANS)

The most immediate response to a stressor is modulated by the autonomic nervous system (ANS)—through its parasympathetic and sympathetic functions—which plays an important role in maintaining physiological homeostasis. The sympathetic discharge, during a "fight-or-flight" response, is accompanied by stimulation of the sympatho-adrenomedullary system, which is pivotal for rapid changes in physiological state, such as increased heart rate and blood pressure by catecholamine–induced excitation of the cardiovascular system (39, 40), whereas parasympathetic activation modulates the sympathetic system and restores the body to a restful state.

During the third trimester, the fetal ANS is changing rapidly (41). The maturational changes in the ANS are pivotal for the successful adaptation of the newborn to extrauterine life. The vagal nerve plays an important role in the parasympathetic regulation of autonomic functioning and subsequent socio-emotional function (42). There are two major components of the parasympathetic nervous system (PNS), namely, the unmyelinated vagal fibers [dorsal nucleus of the vagus (DMNX)] and the myelinated vagal system (nucleus ambiguus). At 9 weeks of gestation, the DMNX is distinguishable from the caudal brainstem, with the first differentiation into two subnuclei (i.e., dorsomedial and ventral) at 13 weeks. At 15–21 weeks, the subnuclei of the DMNX become more clearly visible, and the cytoarchitectonic differentiation of the DMNX is largely completed by 25 weeks (43, 44). Importantly, the phylogenetically primitive unmyelinated vagal nerve does not have many functions

prior to birth, as it facilitates immobilization to deal with environmental challenges.

The nucleus ambiguus system develops later in fetal development, as it heavily depends on myelination. The nucleus ambiguus system is the primary vagal inhibitory pathway, facilitating an active vagal brake, modulating cardiac output by regulating inhibitory vagal control (45). Neurons in the nucleus ambiguus first appear at 8–9 weeks of gestation, with differentiation of neuronal subgroups appearing at 10 weeks of gestation (46). The number of myelinated vagus fibers increases rapidly from 24 weeks of gestation until the first year postpartum, indicating maturation of the parasympathetic branch of the ANS (47, 48). Advanced gestational age is accompanied by increased fetal heart rate variability (HRV) (49); these findings are in line with a rapid maturation of cardiac neuroregulatory activity and especially increasing parasympathetic tone in the last prenatal period (>32 weeks of gestation) (50).

ANS functioning is not solely mediated by the (un)myelinated vagus nerve, autonomic state is also regulated by sympathetic functioning. However, the maturation of the sympathoneural branch is less well-described. It is theorized that the sympathetic system develops before the NA, but after the DMNX (47). In line with this, research does indicate a steady maturational increase with growing fetal age from the late second into the early third trimester [∼16–28 weeks of gestation (51, 52)].

Throughout most of gestation, the medulla (i.e., inner layer of adrenal gland) is not recognized as a distinct structure. At a later stage of development, after the first postnatal week, the adrenal medulla starts to form. Importantly, it takes 12–18 months for the medulla to become adult-like (53).

The fetal development of autonomic control is complex and difficult to disentangle, and important maturational milestones are reached during the transitional period from the second into the third trimester (see **Figure 1**). It is likely that exposure or experiences of stress during those important milestones have a potent effect on ANS development and function, which emphasizes the possible detrimental effects of preterm birth on the programming of ANS and subsequent stress-regulation [*see paragraph on autonomic nervous system (ANS)*].

Fetal Development of the Hypothalamic-Pituitary-Adrenal Axis (HPA-Axis)

Activation of the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) results in the secretion of glucocorticoids, i.e., cortisol in humans, from the adrenal cortex (39), which acts on several organ systems to mobilize energy reserves. The emerging fetal HPA-axis undergoes large shifts in maturation and organization during the prenatal period. The fetal hypothalamus could be longitudinally subdivided into three zones, namely the *midline*, *core*, and *lateral* zones (54, 55). Early gestation (9–14 weeks of gestation) is distinguished by differentiation of the lateral hypothalamic zone, leading to the formation of the lateral hypothalamic area (LHA) and the perifornical hypothalamus. The hypothalamic *core* is differentiated around the second trimester, 18–33 weeks of gestation. The late second until third trimester (24–33 weeks of gestation) is characterized by advances in structural maturation of the periventricular (or *midline*) zone, followed by differentiation in (1) the suprachiasmatic [i.e., circadian clock under the strong influence of light/dark input (56)], (2) arcuate [i.e., sensor to modulate cortisol release (57)], and (3) paraventricular nuclei [i.e., promotes corticotrophin releasing hormone (CRH) and vasopressin (AVP) (58)]. These maturational changes extend into the third trimester. Around the postnatal period (immediate after birth), the major hypothalamic structures are clearly differentiated and resemble an adult-like form (54, 55).

The fetal pituitary gland seems to mature before the adrenal cortex. More specifically, the kidney-shaped anterior lobe of the pituitary gland, which is connected to the hypothalamus, starts to form from Rathke's pouch by 4–5 weeks of gestation. The first 12 weeks of gestation are characterized by major cellular differentiation (59), and by 21 weeks a further distinction can be made between the long and thin stalk region of the pituitary and the posterior lobe (60). Although the pituitary gland matures until the third postnatal month, after which it appears to be adult-like (61), fetal adrenocorticotrophin hormone (ACTH) is detectable by 8–10 weeks of gestation, peaking between the first and second trimester, after which it declines late in gestation (62).

Starting at the 4th weeks of gestation, the adrenal cortex (i.e., outer layer of adrenal gland) begins to form, and the morphology remains relatively stable after 10–12 weeks of gestation. Research suggests that the fetal adrenal cortex is unable to synthesize cortisol between 16 and 22 weeks of gestation, as the 3ß -hydroxysteroid dehydrogenase (3ß -HSD; converts pregnenolone to progesterone) enzyme is not expressed before the start of the third trimester (63). Hence, these findings indicate that the fetus is able to adapt to environmental changes and to maintain homeostasis, through glucocorticoid secretion, after 23 weeks of gestation. However, there is not much consensus on when exactly the fetal adrenal cortex is able to synthesize cortisol. More specifically, at 30 weeks of gestation, the fetal adrenal cortex resembles the elementary form of the adult adrenal cortex [(64), see (65), for review], and some studies suggest that the fetal adrenal cortex is unable to produce cortisol *de novo* until then (66), but instead uses the abundant placental progesterone. This would indicate that in absence of placental progesterone, the fetus might be unable to produce cortisol before 30 weeks of gestation.

The developmental trajectories of the HPA-axis that are established during the prenatal period could have lifelong consequences for future development (see **Figure 1**). Preterm-born infants are neuroendocrinologically immature, and their NICU stay associated with (multiple) stressful events might disturb the central regulation of HPA-axis. Therefore, prematurity might be characterized by the inability to maintain homeostasis in the face of acute stress [see *Hypothalamic-Pituitary-Adrenal axis (HPA-axis)*].

Fetal Development of the Stress-Related Neural Networks

There are three core neural networks that are implicated in the central response of stress, namely the (1) default mode network

[DMN; which includes the posterior cingulate cortex (PCC), hippocampus, and parahippocampal cortex, amongst others (67)], the (2) salience network [SN; which includes the dorsal anterior cingulate cortex (ACC), frontoinsular cortex, amygdala, and several other (sub)cortical structures (68)], and (3) executive control network [ECN; which includes dorsolateral prefrontal and parietal regions (69)]. The ability to dynamically shift neural resources within these large-scale networks is theorized to facilitate adaptive responses to stress, and alterations in these networks possibly underlie phenotypic abnormalities (70). Interestingly, nodes within these networks start to develop in the fetal period, showing large morphological and functional changes throughout gestation. Below we will give a short overview of the typical maturational changes in the embryonic and fetal period (see **Figure 1**).

Default Mode Network

The *hippocampus*, a core node of the DMN, plays an important role in the regulation of the stress response due to its high expression of glucocorticoids and mineralocorticoids, thereby exerting negative feedback on the HPA-axis (71). As early as 9 weeks post-conception, four distinct hippocampal layers can be distinguished: intermediate zone, ventricular zone, hippocampal plate, and marginal zone (72). An unfolded hippocampus, along the medial surface of the temporal lobe, is present at 13 weeks of gestation. Throughout the following weeks, infolding of the hippocampus into the temporal lobe start as the dentate gyrus and cornu ammonis develop into an interlocking C shape. By 18–21 weeks of gestation, the hippocampus shows morphological maturity similar to that of the adult brain (73). The absolute volumes increase linearly from 14 to 22 weeks of gestation, with, relative to other brain regions, a faster growth from 14 to 17 weeks,]but a slower growth from 18 until 22 weeks (73, 74). The dentate gyrus develops latest, showing a mature cytoarchitercute after 34 weeks of gestation (72). Compared to other brain structures, the hippocampus seems to be one of the earliest developing brain regions in humans. Importantly, the morphological development of the *PCC* and *parahippcampal gyrus*, both important components of the DMN, are not well-documented.

Important connections of the adult DMN are already present in the fetal period. For instance, some short-range pathways between the hippocampus and cortico-cortical regions, i.e., entorhinal cortex, are established as early as 19 weeks of gestation (75). Functional studies reported that from 19 weeks onwards, connectivity of the PCC became increasingly negative, which according to the authors, might serve a foundational role in the establishment of large-scale neural networks (76). Similarly, older (>35 weeks of gestation) but not younger fetuses showed a more synchronized positive functional connectivity between the PCC and medial PFC, and negative connectivity to the lateral prefrontal and parietal regions (77). Although the DMN becomes more synchronized across the first 2 years of life and achieve adult-like structures at the end of the first year, including increased connectivity between the PCC and hippocampus, the network is still rather immature in neonates (78–80). In sum, much of the foundation of the DMN is laid down in the early fetal and neonatal period. With the unfolding of several neuromaturational processes, disturbances in normative brain development, including an adverse extra-uterine environment, has likely far-reaching consequences. More studies are needed to elucidate the structural and functional milestones of the DMN and the impact of neonatal stress.

Salience Network

The *amygdala*, a core node of the SN, is a key component of the limbic system and is commonly implicated in emotional and behavioral regulation. This structure shows large morphological changes during fetal development. Differentiation of the amygdala nuclei continuous from the embryonic through the fetal period and neurogenesis is completed by birth (7.5–34 weeks gestation). More specifically, at 12 weeks of gestation migration of the neurons to the lateral amygdaloid nucleus are visible (81), and all major nuclei are formed by 15 weeks. The amygdala appears to be fully mature and functional at birth (81–83), and its connections are laid down early in gestation. Despite the absence of myelin, at a very early stage (i.e., 13–22 weeks of gestation) the amygdala establishes the first connections to several areas of the cortex (84, 85) with the appearance of association white matter fibers, such as the uncinate fasciculus [i.e., a major white matter fiber tract connecting the anterior temporal lobe and the amygdala to the lateral orbitofrontal cortex to the inferior frontal cortex (86)], appearing at around 15 weeks of gestation.

Although structural amygdala connectivity appears early in fetal development, these connections are predominantly short-range, with long-range tracts becoming more evident by term (87). Similarly, the functional connections of the amygdala stabilize early in fetal development. Late second and early third trimester (21st−26th weeks of gestation) are dominated by occipital and temporal connections, with a substantial increase in functional connectivity between the frontal and temporal lobes later in gestation [29–37 weeks of gestation (88)].

The *thalamus* is another core node of the salience network, which is a region that is strongly connected to the amygdala and involved in the regulation of stress, amongst others (89). From 8th week on, thalamic neurons show intensive morphological changes, with projection from the spinal cord to the thalamus. From 10 to 14 weeks of gestation, neuronal differentiation into several thalamic nuclei begins (90). Neurogenesis in the posterior medial thalamus extends into the late first and early second trimesters of pregnancy. By 26 weeks' gestation, the characteristic layers of the thalamus are visible, with obvious similarities to the adult brain. Thalamocortical pathways to the subplate neurons are evident at 17 weeks of gestation, but the thalamic projections to the cortical plate continue to develop later during the fetal period (24–32 weeks) (84, 85, 87, 91), reaching adult-like connections at 34 weeks of gestation.

It is well-known that subcortical structures demonstrate earlier maturation than the cerebral cortex. However, the *insular cortex*, also described as the "center of salience processing" (92), is among the first macroscopical structures that can be identified in the human fetal cortical development. Afif et al. (93) described the morphological stages of insular sulci and gyri maturation, with the first sulcus appearing at 13–17 weeks of gestation.

Around 27–28 weeks of gestation, all insular sulci and gyri are in place and its structure is similar to its adult-like form. More specifically, by the end of the third trimester, the insula can be divided into two parts, the anterior insula (i.e., comprises three short gyri), and the posterior insula (i.e., comprises two long gyri). Radial migration pathways, between the ventricular zone and superior temporal region, were observed at 15 weeks of gestation, continuing to grow in number and thickness until 20 weeks of gestation (94). Further, by 26 weeks of gestation, migration pathways are regressing, and by 31 weeks of gestation the insular neuronal migration is fully completed. Importantly, similar connectivity is observed between 31 and 40 weeks of gestation, and it seems that several pathways observed in the fetal period (e.g., insular-parietal and insular-temporal pathways) are similar to those in adults.

The fact that several core nodes of the salience network mature before birth emphasizes the importance of early fetal development for functioning of the salience network. To date, there are only a few studies directly investigating early development of structural and functional connectivity within the salience network. One study did report synchronous activity of the anterior insula with anterior cingulate cortex in neonates, although quite primitive (95). Interestingly, however, while the neonatal brain consisted of large locally connected clusters, 1- and 2-year olds demonstrated more sophisticated distributed topology. Further, enhanced connectivity between the anterior insular and long-range prefrontal cortices and anterior cingulate cortex seemed adult-like in 1-year olds (with only a few changes in 2-year olds) (95). In line with this, other studies do suggest that connection strength increase with age, but only moderately, leading to still premature network topologies at the end of the first year (80) and second-year (96). Interestingly, recent studies were able to identify so-called "hubs," which are highly connected regions, in the fetal period. Both the temporal lobe (97) and the insular cortex (76, 98) were found to be already highly connected before birth. In sum, key nodes of the salience network seem, on a morphological level, adult-like at birth, but the topological features of fetal brain network remain underdeveloped (see **Figure 1**). Changes in typical fetal development, such as preterm birth, might precociously impact the architectural characteristics of the immature SN.

Executive Control Network

Brain regions that serve a high-order function, such as the *dorsomedial prefrontal cortex* (DMPFC) and dorsal *posterior parietal cortex* (DPPC), which are all core nodes of the ECN, mature latest. The corticogenic events occur at different rates in different regions, and some cortical areas start to differentiate earlier in gestation than others. For instance, studies consistency showed a maturational lag in the frontal cortical regions, as the neural migration [i.e., as indicated by peak fractional anisotropy [26–30 weeks of gestation]] of the frontal lobe is preceded by the parietal lobe (21–25 weeks gestation) [e.g., (99, 100)]. Some studies suggest that the superior longitudinal fasciculus (SLF), a white matter tract connecting the superior parietal and superior frontal lobes and extending to the dorsal premotor and dorsolateral prefrontal regions, is completely absent in the fetal brain (84, 101). However, a recent study was able to reliably visualize the SLF at 26 weeks, suggesting that the SLF may start to develop in the second trimester, and continue to develop throughout gestation (102). In line with this, short- and long-range corticocortical association pathways could be observed at 22 weeks of gestation, and become more prominent throughout gestation (103).

Short-range cortico-cortical tracts emerge prior to gyrification in regions where sulci will later develop. The cortical plate starts to form in the human telencephalon around 7–10 weeks of gestation (104). The timing of the different types of sulci occurs hierarchically. Primary sulci start to appear as early as 10 weeks of gestation and continue to develop until the 28th week of gestation (105, 106). This is followed by the development of secondary and tertiary sulci. All the primary and most of the secondary sulci are believed to be present by 34 weeks of gestation (106, 107), whilst tertiary sulci appear around term [36–41 weeks of gestation (108)]. A core node of the ECN, the *superior frontal sulcus*, emerges between 22 and 24 weeks of gestation (109–111), and become clearly visible from ∼27 weeks onwards (105, 106, 112).

Although the ECN is observable in fetuses and neonates (see **Figure 1**), it is still in a premature form at the end of the first postnatal year (79). However, the fetal period constitutes a time of vast development, and a study on functional connectivity found large differences in the ECN between younger and older fetuses. More specifically, they reported that older fetuses (i.e., 34 weeks of gestation) showed increased connectivity between the prefrontal areas and the parietal cortex, compared to younger fetuses (i.e., ∼27 weeks of gestation) (76). In sum, using structural and functional connectivity and graph-theoretical analyses, studies were able to provide preliminary evidence for the emerging ECN in fetuses, highlighting the importance of the establishment of ECN nodes to the effect of functional and structural injuries typically sustained during premature birth. Importantly, the large variability in spatiotemporal development of nodes seem to indicate a developmental sequence starting from the DMN, to the SN, and finally the ECN, as the (sub)cortical nodes implicated in the DMN and SN appear to be maturing during the beginning of the first trimester, as opposed to the relatively delayed maturation of cortical nodes implicated in the ECN.

NEONATAL STRESS AND BRAIN DEVELOPMENT

More than 50 years ago, researchers expressed their concerns regarding the possible detrimental effects of neonatal stress on physiology and behavior, stating that early experience in animals, such as handling or electric shock, lead to changes in corticosterone response and emotionality [e.g., (113–115)]. Rodent studies gave rise to a large body of experimental studies reflecting the importance of early neonatal stress in regulating brain development (116, 117). The studies that have been conducted thus far found quite consistently that, similar to animal studies [e.g., (118)], exposure to neonatal stress is associated with alterations in several structures. For instance,

using the Neonatal Infant Stressor Scale [NISS; cumulative stress score including physical and environmental stressors (25)], researchers found an association between high stress and decreased frontal and parietal brain volumes, as well as alterations in white matter microstructure in the temporal lobes in preterm born infants at term equivalent age (119). Similarly, the number of invasive procedures was associated with reduced white matter and subcortical gray matter maturation in preterm neonates. Moreover, greater invasive procedures were independently associated with reduced total brain volume, white matter diffusivity, and maturation of subcortical gray matter (e.g., thalamus, basal ganglia) (49, 120). Interestingly, Brummelte and colleagues could distinguish a period of increased vulnerability, namely early procedural pain [i.e., before scan 1 (median 32.1 weeks of gestation)] had a stronger association with white matter maturation compared to later stress (around term equivalent age), whereas subcortical gray matter showed sustained sensitivity toward neonatal stress (120). These results are in line with findings from another study, where they reported that functional and structural connectivity patterns were affected following exposure to early (i.e., from birth to scan 1), but not late neonatal stress [i.e., scan 1 to scan 2 [term-equivalent age]]. More specifically, early neonatal stress (e.g., heel lances, central line insertion) was associated with weaker structural (121) and functional (122) connectivity between the right insula and limbic system, and in the thalamocortical pathways. However, some inconsistencies remain, as researchers also reported no association between neonatal stress and hippocampal growth (123). With some ambiguity, these studies converge to reveal a developmental period of increased sensitivity to stress, subsequently affecting stress-regulatory networks. However, there have been no further studies to confirm the possible detrimental effects of postnatal stress on large-scale brain networks, and the possible increased risk of future social and affective functional impairment.

The adverse effects associated with exposure to postnatal physical stress appear to extend beyond the relationships observed in the neonatal period. For instance, a higher number of invasive procedures during NICU admission was related to abnormalities in white matter microstructure (e.g., superior white matter), as reflected by an increase in radial diffusivity at age 7 (124). Procedural pain was also observed to be related to abnormal maturation of brain volumes at school age, including the hippocampus, amygdala, thalamus, striatum, globus pallidus, and cerebellum (125, 126), and lower cortical thickness (127). Studies using magnetoencephalagraphy (MEG) found that differences in spontaneous gamma- to alpha-band oscillations in preterm-born school-age children were predicted by the number of invasive neonatal procedures, which, as suggested by the authors, might be attributed to alterations in thalamorcortical connectivity (128, 129). Hence, the abnormal maturation observed in the neonatal period seems to persist into childhood, affecting brain regions implicated in the regulatory capacity to future stressors (see **Table 1** for an overview).

These findings are supported by evidence from fetal stress models demonstrating that full-term and preterm neonates exposed to prenatal stress showed alterations in brain development. More specifically, prenatal stress (e.g., maternal anxiety/depression) was associated with reductions in region-specific gray matter volume [i.e., PFC, temporal lobe (140)], alterations in white matter microstructure (e.g., amygdala, limbic system) (141–143), and reduced functional and structural connectivity between the amygdala, limbic, and frontal regions in infants (144–146). The effects of prenatal stress on brain development have been extensively reviewed elsewhere [see (147–149)].

Although changes in previously described brain regions have been consistently implicated in a wide range of behavioral problems in preterm born individuals [e.g., (150–155)], it remains elusive whether alterations in brain development might modulate the relationship between neonatal stress and future affective and social functioning.

MECHANISMS UNDERLYING LATER LIFE RESILIENCE AND VULNERABILITY FOLLOWING PREMATURITY

Autonomic Nervous System (ANS)

Ex utero third trimester development often leads to alterations in normal autonomic development in extremely preterm infants, which is essential for respiratory and cardiovascular homeostasis (156). General maturation of the ANS is often assessed using indices of heart rate, blood pressure, and respiratory rate. Studies showed an impaired autonomic maturation in preterm born neonates, as reflected by dampened sympathetic (e.g., low-frequency HRV) and parasympathetic (e.g., high-frequency HRV) tone (41, 50, 157, 158). Thus, far only one study investigated the sympatho-adrenomedullary system (SAM), with results indicating elevated sympathoadrenal tone (as indicated by increased levels of catecholamines) in preterm-born children (159). This increased release of catecholamines could be attributed to the reduced parasympathetic inhibition, as mentioned previously.

The autonomic development is altered during the neonatal period. Accordingly, an increasing body of evidence suggests that ANS dysfunction following prematurity persists into infancy (160, 161), childhood [(162, 163); but not all (164)], adolescence (165), and adulthood (166). This prolonged abnormal maturation of ANS functioning could be attributed to the amount of neonatal stress. More specifically, research showed that greater exposure to neonatal stress was related to dampening of ANS reactivity (130, 131).

Changes in central autonomic regulation in typically developing individuals limit the capacity to adequately respond to environmental changes, and have previously been implicated in psychiatric disorders (167–169). Consistent with this, ineffective vagal modulation has been implicated in dysfunctional emotion regulation in toddlers [e.g., (170)], children [e.g., (171)], and adults [e.g., (172)]. Interestingly, similar findings were observed in preterm infants, namely preterm born infants' degree of respiratory sinus arrhythmia (RSA) was positively associated with their social competence, whereas lower (mean) heart rate was associated with less behavioral problems and

TABLE 1 | Neonatal stress and neurobiological systems.

References	Population	Time of assessment	Sample size (N)	Stress measure	Outcome
Brain development					
Brummelte et al. (120)	Infants (born 24–32 weeks)	32 and 40 weeks	86	Number of invasive procedures: early (birth-scan 1) and late (scan 1-scan 2).	Greater invasive procedures: ↓ white matter FA, ↓ subcortical gray matter NAA/choline. Effects dependent on timing stress.
Chau et al. (125)	Children (born <32 weeks)	8 years of age	57	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓amygdala volume, ↓ thalamus volume. Stress × COMT ↓ hippocampal subregional volume
Doesburg et al. (128)	Children [born extremely preterm [24–28 weeks], very preterm [28–32 weeks], and full-term]	8 years of age	54	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: atypical spontaneous neuromagnetic activity (*only in extremely preterm born children*)
Duerden et al. (123)	Infants [born very preterm [<33 weeks]]	32 and 40 weeks	138	Number of invasive procedures during the stay in the NICU: categorized into two groups	Greater invasive procedures: no association with hippocampal growth
Duerden et al. (121)	Infants [born extremely preterm [24–28 weeks] or very preterm [29–32 weeks]]	32 and 40 weeks	155	Number of invasive procedures: early (birth-scan 1) and late (scan 1-scan 2)	Greater invasive procedures: ↓ lateral thalamus volume, ↓ metabolic growth (NAA/Cho), ↓ FA corpus callosum, posterior white matter, cingulum, and fornix. (*only in extremely preterm born children in combination with early stress*)
Kozhemiako et al. (129)	Children [born extremely preterm [24–28 weeks], very preterm [29–32 weeks], and full-term]	8 years of age	100	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: atypical spontaneous neuromagnetic activity (*only in extremely preterm born children*)
Ranger et al. (127)	Children [born very preterm [27–32 weeks]]	8 years of age.	42	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓ cortex thickness (e.g., frontal, parietal, and temporal regions)
Ranger et al. (126)	Children [born very preterm [27–32 weeks]]	8 years of age	42	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓ cerebellar volumes
Schneider et al. (49)	Infants [born very preterm [<30 weeks]]	29, 31, and 40 weeks	51	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓ growth thalamus, basal ganglia, total brain volumes
Smith et al. (119)	Infants [born very preterm [<30 weeks]]	Term equivalent age	44	Neonatal Infant Stressor Scale: during stay in the NICU or until term equivalent age	Greater number of stressors: ↓ frontal and parietal diameter, and ↓ interhemispheric connectivity temporal lobes
Tortora et al. (122)	Infants [born very preterm [<33 weeks]]	Term equivalent age	46	Number of invasive procedures: categorized into four groups	Greater invasive procedures: ↓ connectivity thalami—bilateral somatosensory cortex, ↓ connectivity insular cortex—ipsilateral amygdala/hippocampus
Vinall et al. (124)	Children [born very preterm <33 weeks)	7 years of age	50	Number of invasive procedures during the stay in the NICU	Greater number of stressors: ↓ white matter FA
ANS function					
Goffaux et al. (130)	Children [born very preterm [<33 weeks], and full term]	7–11 years of age	26	Total number of days spent in the NICU and total numbers of days spent under mechanical ventilation: categorized into two groups	Greater invasive procedures: no changes in heart rate and pain sensitivity in high-stress group in response to conditioning cold stimulation
Grunau et al. (131)	Infants [born very preterm [<33 weeks]]	32 weeks	136	Number of invasive procedures from birth until time of assessment	Greater invasive procedures: ↓ autonomic reactivity in response to blood collection
HPA axis function					
Brummelte et al. (132)	Children [born extremely preterm [<28 weeks], very preterm [<32 weeks], full term]	7 years of age	129	Number of invasive procedures from birth until term equivalent age	Greater invasive procedures: ↓ basal cortisol (study day and at home)

(Continued)

TABLE 1 | Continued

References	Population	Time of assessment	Sample size (N)	Stress measure	Outcome
Grunau et al. (133)	Infants [born extremely preterm [<28 weeks], very preterm [<33 weeks], and full term]	8 months	76	Number of invasive procedures from birth until term equivalent age	Greater invasive procedures: ↑ sustained basal cortisol (*only in extremely preterm born infants*)
Grunau et al. (134)	Children [born very preterm [<33 weeks] and full term]	7 years of age	128	Number of invasive procedures from birth until term equivalent age	Greater invasive procedures: ↓ hair cortisol (*stress×NFKBIA effect, only in boys*)
Provenzi et al. (135)	Infants [born very preterm [<33 weeks] and full term]	3 months of age	90	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓ cortisol reactivity to still-face procedure
Epigenetics					
Chau et al. (136)	Children [born very preterm [<33 weeks] and full term]	7 years of age	111	Number of invasive procedures during the stay in the NICU	Greater invasive procedures: ↓SLC6A4 methylation (*only in children with COMT Met/Met genotype*)
Fumagalli et al. (137)	Infants [born very preterm [mean of 30 weeks]]	Birth and NICU discharge	56	Principal component analysis on number of invasive procedures	Greater invasive procedures: ↑ delta SLC6A4 methylation
Montirosso et al. (138)	Infants [born very preterm [<33 weeks] and full term]	Birth and NICU discharge	78	NICU stay: difference score between birth and NICU discharge	↑ delta SLC6A4 methylation at discharge than at birth
Provenzi et al. (139)	Infants [born very preterm [<33 weeks] and full term]	Birth and NICU discharge	88	Number of invasive procedures during the stay in the NICU: categorized into two groups	Greater invasive procedures: ↑ delta SLC6A4 methylation

↑ *increase(d);* ↓ *decrease(d).*

greater social competence (173). These findings indicate that functional deficiencies of the vagus, and more specifically the phylogenetically newer ventral vagal complex (VVC; i.e., part of the nucleus ambiguus and suppresses robust emotional reactions), might underlie difficulties in emotion regulation in preterm-born individuals (see **Table 1** for an overview).

Although no further studies investigate the relationship between ANS functioning and outcome, these findings conform to the framework of the *polyvagal theory*, proposed by Porges (45, 174–176), which states that alterations in vagal tone and reactivity, and thus parasympathetic regulation, possibly lead to the development of psychiatric disorders in preterm-born individuals. The framework articulates three phylogenetic stages that underlie different behavioral responses, all associated with a distinct autonomic subsystem: (1) social communication [e.g., emotion (via ventral vagal complex)], (2) mobilization [e.g., fight-flight responses [via sympathetic-adrenal system]], and (3) immobilization [e.g., tonic immobility (via unmyelinated vagus)]. Alterations in these distinct subsystems possibly underlie the behavioral problems observed in preterm individuals. However, further research is needed to delineate the effects of preterm birth, and postnatal stress, on autonomic control and subsequent longitudinal brain and behavioral development.

Hypothalamic-Pituitary-Adrenal Axis (HPA-Axis)

A considerable array of research has found that neonatal adversity impacts neuroendocrine development. Studies reported, for instance, that more invasive procedures were associated with lower cortisol responses to a stressor [below 33 weeks post-conception (133); for a review see (177)]. Also, hyporeactivity to socio-emotional stress, as measured with the Face-to-Face Still-Face (FFSF) procedure (i.e., assesses socio-emotional regulation by rating negative emotionality, social engagement, and avoidance behavior), has been linked to the number of invasive procedures during NICU admission in 3-month old preterm infants (135). In children born very preterm, greater exposure to neonatal pain-related stress was associated with higher basal cortisol levels at 8 months (133) and 18 months (178), but lower basal, diurnal (132) and cumulative (hair) cortisol (134) at age 7–8 years. It is well-recognized that chronic stress can lead to downregulation of cortisol production [e.g., (179)], thereby reducing the detrimental effects of glucocorticoids. These alterations in HPA-axis functioning seem to persist into adulthood. More specifically, studies showed both decreased and increased HPA-axis responses (i.e., cortisol and ACTH) following acute stress, when compared to full-term controls (180, 181). These mixed results might be due to the developmental timing at which neonatal stress occurs, including age of onset, duration, and severity, affecting the effects of concurrent stress on the dynamically changing HPA-axis (*see paragraph on fetal development of the hypothalamic-pituitary-adrenal axis*).

The different developmental stages seem to be mirrored by a shift between hypo- and hyper-reactivity of the HPA-axis, with postnatal stress possibly altering the set-point of HPA-axis functioning in preterm-born individuals. This observation highlights the fact that both the type and magnitude of the stress-responses largely depends on the timing of the stressor. Moreover, exposure to postnatal stress at different points in the

development of the HPA-axis might exhibit a different impact. Indeed, rodent studies showed that "early" maternal separation (i.e., 3–4 postnatal days) was associated with a hyper-responsivity to stress later in life, while "late" maternal separation (i.e., 7–8 or 11–12 postnatal days) showed an effect in the opposite direction (182, 183). These findings are in line with studies indicating that synthesis of CRH receptors is regionally distinct and age-specific. More specifically, CRH receptor density in rodents is highest during early postnatal days (i.e., 2–9), with CRHR1 mRNA levels increase to a maximal of 300–600% of adult levels (184). Perturbation of the profound changes in CRF system, due to extreme and chronic stress, might have long-lasting consequences for development. Hence, these critical developmental processes are extremely complex, and dependent on the timing of exposure, postnatal stress might have differential consequences. However, to date, this explanation for the mixed results of HPA-functioning in preterm born individuals remains speculative, and research is needed to delineate the possible time-specific effects of postnatal stress following preterm birth.

Surprisingly, altered HPA-axis functioning and its impact on (brain) development in ways that increase susceptibility to later stress-related disorders have not been extensively studied in preterm born individuals (see **Table 1** for an overview). Thus far, higher basal cortisol in preterm born infants has been associated with poorer mother interactive behavior, as well as more problems in terms of emotional reactivity, anxiety, depression, and attention, amongst others (185). Similar findings were observed in preterm-born children, that is, increased HPA-reactivity was linked to more problems with attention, emotional reactivity, anxiety, depression, and negative mother-child interactions (186). Hence, converging evidence does suggest that HPA-axis functioning is a key mediator of developing psychopathology. Although the structural and functional development of neural networks is tightly linked to HPA-axis functioning, such that early life adversity sensitizes hippocampal-amygdala responses to acute stress (187, 188), to date, there are no studies that explored neural circuitry associated with HPA-dysfunction in preterm born individuals.

In sum, postnatal stress following prematurity lead to long-lasting changes in HPA-functioning, which, in turn, is associated with problem behavior (e.g., emotional reactivity, anxiety). Differences in HPA-functioning might be attributed to the timing of postnatal exposure, although extensive research is needed to disentangle the neurobiological mechanisms involved. Importantly, there is currently little research as to whether the altered patterns of HPA-axis functioning in the context of prematurity and postnatal stress are permanent, what epigenetic pathways might underlie the contradicting findings (*see paragraph on epigenetic pathways*), and the potential of prevention following postnatal interventions such as skin-to-skin contact (*see paragraph on possibilities for early intervention*).

Epigenetic Pathways

There is increasing evidence for the role of genetic and epigenetic variation in long-term effects of early life stress (189). It is suggested that epigenetic markers are developmentally sensitive to the quality of the pre- and post-natal environment and that early adversity produces lasting epigenetic modifications (190–192). Studies on the so-called "early-life programming" of the epigenetic regulation of gene transcription, have mainly focused on the serotonin transporter, due to its polymorphisms and role in mediating early stress and later life mental health, and glucocorticoids (GR), due to its negative feedback control on stress responsivity [for a review see (193)]. Importantly, most studies have focused on candidate genes related to serotonin and glucocorticoid functioning. Below we will review evidence from postnatal studies, but we will rely on prenatal studies where investigation in preterm individuals is lacking.

There are a few studies that investigated the influence of postnatal stress on *SLC6A4* [i.e., a gene encoding the serotonin transporter (*5-HTT*)] promoter methylation. Studies reported an association between greater postnatal stress and lower *SLC6A4* methylation in preterm-born infants (138, 139) and school-aged children with the *COMT 158 Met/Met* genotype (136). Importantly, authors suggested that *SLC6A4* promoter methylation could not be attributed to preterm birth *per se*, rather, high levels of postnatal stress exposure altered the transcriptional functionality of *5-HTT* (139, 194). In turn, greater *SLC6A4* methylation predicted poorer stress-regulation in response to the still-face procedure at NICU discharge (194). Additionally, methylation at discharge was associated with greater negative emotionality and suboptimal socio-emotional regulation (137, 194). Thus far, only one study investigated the possible moderating role of *SLC6A4* methylation on the relationship between NICU-stress and later brain development. More specifically, authors reported that preterm infants exposed to greater stress showed higher *SLC6A4* methylation, which in turn was associated with reduced anterior temporal lobe (ATL) volumes (137).

Extensive scientific literature has repeatedly reported that changes in glucocorticoid receptor methylation (*NR3C1*) play a pivotal role in the regulation of the HPA-axis and the endocrine response to stress (195). Indeed, infants exposed to third-trimester prenatal stress, as measured by maternal emotional state, showed increased methylation of the *NR3C1* gene (196, 197). Additionally, Oberlander and colleagues found that increased methylation of the *NR3C1* was in turn associated with increased HPA-axis reactivity. Even though *NR3C1* is tightly linked to stress vulnerability and resilience, studies investigating the epigenetic changes in *NR3C1* following prematurity is limited. Few studies did investigate the role of prematurity in *NR3C1* methylation, and findings were in line with studies on prenatal stress [for a review see (193, 198)], that is, preterm infants exposed to an adverse postnatal environment influenced *NR3C1* methylation (199). Specifically, increased methylation of glucocorticoid receptor gene was observed in the first 4 days following preterm birth. However, findings remain inconsistent as studies demonstrated both decreased and increased DNA methylation of *NR3C1* in high-risk preterm infants [i.e., scoring high on Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS) or more medical problems] compared to low-risk preterm infants (200, 201).

Several other genes have been found imperative for the regulation of HPA-axis function, including the *FKBP5* gene, which exerts an inhibitory role on GR signaling by modulating hormone-binding affinity (i.e., the strength of

binding interaction) (202, 203), and the 11beta-hydroxysteroid dehydrogenase type 1 and 2 (*11β-HSD*), functions as a dehydrogenase which degrades cortisol to cortisone (*11β-HSD2*), and catalyzing the conversion of inactive cortisone to active cortisol (*11β-HSD1*) [for a review see (204)]. So far, only one study investigated *FKBP5* gene transcription in relation to prematurity. Piyasena et al. (205) showed that preterm born infants had markedly lower methylation at *FKBP5* compared to term-born infants. Importantly, these differences in *FKBP5* methylation were resolved at 1 year of age. In line with this, a longitudinal epigenome-wide association study (EWAS) identified a total of 1,555 sites with significant differences in methylation in preterm born infants, but the majority of these differences did not persist into adulthood (206). Hence, these studies question whether the effects of prematurity and postnatal stress on DNA methylation persist across the life course.

11β-HSD1 is seen in the adult central nervous system and has previously been shown to influence HPA-axis regulation (207, 208), whereas *11β-HSD2* has a major central role in developmental programming due to the high expression in placenta and fetal tissue (209). As the function of *11β-HSD* seems to reflect protection from the deleterious consequences of glucocorticoid overexposure, it has been suggested that *11β-HSD* might function as a potential pathway in early life stress exposure and later outcome (210, 211). Studies indeed showed that prenatal stress was associated with downregulation of placental *11β-HSD2* gene encoding (212, 213), as well as lower and greater methylation of *11β-HSD2*, respectively (214, 215). In turn, in rodents, *11β-HSD2*$^{-/-}$ selectively determined programming of anxiety and depressive-like adult behavior (204, 211, 216). Similarly, *11β-HSD1*$^{-/-}$ mice showed elevated basal corticosterone levels and exaggerated responses to stress (207, 208, 217). Increased levels of placental *β-HSD1* mRNA were observed in mothers exposed to prenatal stress (218, 219), leading to increased glucocorticoid transport to the fetus. Although these findings could theoretically be extrapolated to a preterm population, as the assumed changes in *11β-HSD* fail to protect the immature neurons from premature stress exposure, further investigation will be required to determine the degree to which changes in *11β-HSD* are prevalent in preterm born individuals.

Only recently studies began to enhance our understanding of epigenetic changes following prematurity. Converging evidence suggests that preterm born individual show profound changes in glucocorticoid and serotonin transporter gene transcription, with some studies suggesting that these alterations can be specifically attributed to postnatal stress. Other genes involved in the regulation of HPA-axis, such as *FKBP5* and *11β-HSD*, have not been studied extensively. Importantly, epigenetic changes following prematurity might be non-persistent. Nonetheless, more studies are needed to further delineate the epigenetic changes following prematurity and the specific role of postnatal stress. There are several theoretical and methodological challenges in the field of behavioral epigenetics in preterm born individuals, including heterogeneity of the population (e.g., gestational age), lack of prospective longitudinal and epigenome-wide studies, small sample sizes, and inadequate control of confounders (e.g., race), amongst others.

Disruptions in the Neural Equilibrium

It is well-known that stress induces large-scale neural modulations and that extreme and prolonged stress trigger long-lasting changes in network balance. Both the salience network (SN) and the executive control network (ECN) are implicated in the adaptive regulation of stress, and these complex networks already originate in the fetal period (*see paragraph on Fetal development of the stress-related neural networks*). In the face of environmental challenges, via increased catecholamines, the SN is supposedly upregulated, facilitating increased vigilance and attentional reorienting, and autonomic-neuroendocrine control (220). On the contrary, the ECN, which is implicated in cognitive control processes and decision-making, is downregulated following stress (221). It is theorized that in the aftermath of stress, resources are allocated to the ECN, downregulating the SN (70). Disruptions in this neural equilibrium, possibly due to morphological alterations in prefrontal (222) and hippocampal (223) neurons following chronic stress, have been implicated in the pathogenesis of Post-Traumatic Stress Disorder (PTSD) (224, 225), depression (226, 227), anxiety (228, 229), bipolar disorder and schizophrenia (230, 231), amongst others.

Emotion Processing and Executive Functioning

A large number of studies have described a behavioral phenotype in preterm-born individuals constituting problems in the area of socio-emotional and executive functioning [for further details please see (232–234)], two higher-order cognitive phenotypes implicated in stress regulation (235, 236). The neural mechanisms underlying these behavioral phenotypes appear to be altered in preterm-born individuals. Although there are currently no studies investigating the role of postnatal stress on these stress-regulatory neural mechanisms, a growing number of studies does recognize the pivotal role of large-scale neural networks, rather than region-of-interest (ROI) based approaches, in preterm-born individuals.

Resting-state network studies showed an altered coupling between the SN and default mode network (DMN). The DMN comprises the medial prefrontal, posterior cingulate, precuneus, and bilateral angular gyrus (237), and exhibits low-frequency activity at rest, and has been proposed to be related to self-referential mental activity, including task-unrelated imagery and thoughts and self-reflection in preterm born individuals (238). Studies consistently reported a hypo-connectivity between the amygdala, mPFC, posterior cingulate cortex (PCC), anterior insula (AI), and the precuneus (pC) in preterm-born infants at term equivalent age (239), adolescents (240), and adults (241, 242). As suggested by the authors, the negative connectivity between the SN (i.e., AI/amygdala) and DMN (i.e., PCC/pC) could indicate an overactive inhibitory function of the PCC in modulating the left amygdala, greatly affecting their emotion processing (241). In line with this, a study reported that variability in the connectivity between the amygdala and other regions was predictive of greater internalizing symptoms at 2 years (239). Also, alterations in white matter microstructure involved in the SN and other networks, including the thalamus, inferior fronto-occipital fasciculus, and inferior/superior longitudinal fasciculus have been associated

with internalizing symptoms in preterm born children aged 9–16 (243).

Preterm children also showed structural alteration in the anatomical organization of the cortico-basal ganglia-thalamo-cortical pathway (CBGTC). Especially connections between the thalamus, putamen, globus pallidus and caudate nucleus were weaker (151). These SN-specific nodes are disrupted in psychiatric diseases [e.g., PTSD, obsessive-compulsive disorder (OCD), schizophrenia (244)], dissociating multiple interconnected mental operations, such as processing affective content, decision making, and attention (245). Studies also reported significant smaller amygdala volumes in preterm-born infants at term equivalent age, compared to term born infants, and this altered amygdala development has been linked to maladaptive fear-processing (as measured by Unpredictable Mechanical Toy episodes) (246). A key limbic tract, the uncinate fascicle [i.e., temporo-amygdala-orbitofrontal network (247)], critical for social-emotional functions (86, 248), showed significant white matter reductions in preterm-born individuals [children; (249); adolescents (250)]. At present, it would be helpful to have a clearer understanding of how these neural patterns vary as a function of postnatal stress.

These limbic-cortical pathways are not only involved in socio-emotional behavior but also play a pivotal role in higher-order behavioral control. Studies repeatedly reported significant alterations in white matter microstructure, including the cingulum (i.e., connection between anterior cingulate cortex, dorsolateral prefrontal cortex, and inferior parietal lobe), fronto-occipital fascicles (i.e., bridging frontal-temporal-parietal-occipital lobe), fornix, corpus callosum, and superior longitudinal fasciculus, amongst others (153, 251–255). These white matter indices have been directly linked to alterations in executive functioning, that is, lower executive functioning was related to reductions in several white matter microstructure (e.g., inferior-fronto-occipital fascicles, cingulum, and superior longitudinal fasciculus) [Wisconsin Card Sorting Test (256); Test of Everyday Attention for Children (TEA-Ch) (257); Child Behavior Check List (CBCL) (153); Delis-Kaplan Executive Function Systems (D-KEFS) (258)]. Importantly, one study was unable to find an independent effect of preterm birth on white matter microstructure, and authors emphasized that postnatal factors, such as the degree of stress (i.e., days of mechanical ventilation), grossly impact postnatal brain development (259).

The alterations in connectivity are also observed on a functional level. Specifically, studies found that preterm born adults, compared to controls, showed increased activity in the middle temporal/occipital gyrus, posterior cingulate gyrus, and precuneus [go-/no-go task; (260); verbal fluency task (261)], which replicates previous findings (262). Additionally, during oddball trials (i.e., "odd" stimuli to control for low-frequency no-go stimuli), preterm-born young adults displayed attenuated brain activation in a fronto-parietal-cerebellar network. More recent studies disentangled the neural underpinnings of proactive vs. reactive cognitive control in preterm-born adults. Using the Not-X continuous performance test (CPT), authors reported (1) hypo-activation between the frontal pole and anterior cingulate gyrus, as well as the posterior cingulate gyrus and precuneus, and (2) hyper-activation between the posterior cingulate gyrus and precuneus, and the right lateral occipital cortex and angular gyrus (255). In other words, authors showed that preterm born adults exhibited more reactive behavioral control, rather than proactive. Recent research started to reveal the importance of these functional patterns, as well as the decoupling between the ECN and DMN, for the development of cognitive control (263) and emotion-regulation (264).

In sum, preterm born individuals show profound alteration in large-scale brain networks involved in emotion regulation and executive functioning, the SN and ECN, respectively. These changes possibly underlie individual differences in stress-sensitivity, as both large-scale networks, and its behavioral phenotype, have been implicated in adaptive stress responses (70). As mentioned previously, the DMN, SN, and ECN start to develop during gestation. Although these networks are still immature at birth, the formation of important pathways during gestation gives rise to potential points of vulnerability. The degree to which postnatal stress might underlie these extensive network changes in preterm born individuals remains elusive.

OPPORTUNITIES FOR EARLY INTERVENTION

Inadequate treatment of stressors in preterm infants have previously been associated with short- and long-term alterations in brain and behavior, greatly impacting their ability to maintain homeostasis (*see paragraph on neonatal stress and brain development*). This highlights the importance of adequately and promptly assessing stress in the newborn, and gave rise to the Newborn Individualized Developmental Care and Assessment Program (NIDCAP) (265, 266), which is a technique that uses detailed observations of infant behavior to provide caregivers and parents with recommendations on how to minimize stress. Developmental care theories postulate that one should actively observe the infant, during several caregiving procedures (e.g., collection of a blood sample), to assess the infant's efforts of self-regulation in response to stress. Based on such observations, both clinicians and families can make adjustments to optimize and adapt the traditionally delivered newborn intensive care to the infants' current needs. These adjustments can include interventions developed to increase self-regulation in the preterm infant. In full recognition of the fact that there are numerous interventions aimed at preventing or reducing postnatal stress, including the possible protective effects of fetal neurosteroids such as allopregnanolone, and its inhibiting properties in relation to the HPA-axis [(267, 268); for a review see (269)], for the purpose of this review, we decided to focus on non-pharmacological interventions as several analgesics have the potential to adversely impact the developing postnatal brain by altering neuronal processing [e.g., (270, 271)].

Skin-to-skin contact, also called kangaroo care, is a promising intervention possibly reducing infants' stress during NICU admission. For instance, research showed that preterm born

infants exhibited lower basal stress levels (i.e., autonomic responses) during kangaroo care when compared to regular care (272, 273), as well as lower stress reactivity when exposed to physical stress (274–277), and improved white matter microstructural development (278). Kangaroo care also seems to affect HPA-axis functioning, as indicated by reduced saliva cortisol levels when exposed to a period of kangaroo care (279), as well as lower salivary cortisol reactivity at 1 month (280). However, kangaroo care seems to not bring about sustained reductions in salivary cortisol (280–282). Interestingly, research did show sustained effects on autonomic control. More specifically, one study found that kangaroo care accelerated the maturation of vagal tone between 32 and 37 weeks of gestation, as indicated by increased amplitude of RSA, positively affecting autonomic control (283). As suggested by the authors, kangaroo care might exert developmentally sensitive effects and should be administered during an appropriate developmental window to alter the maturational trajectories of systems that are currently developing. In other words, the timing of the intervention is pivotal for achieving optimal levels of physiological maturation. Although kangaroo care showed some positive effect on preterm infants' physiological stability and maturation, the degree to which these effects are long-term remains elusive.

Although inconclusive, there is some evidence for the positive effects of kangaroo care on attachment behavior and parental stress levels. More specifically, kangaroo care positively affected mother's mood, reduced parental stress levels, increased parental affiliative (e.g., touch) and attachment behavior when compared to standard care [e.g., (177, 272, 284, 285)]. These changes in parental care and behavior might, in turn, have positive effects on the preterm infant's stress-regulatory capacities [e.g., (286, 287)], which provide preliminary support for changing the biological organization of the stress system in preterm infants through parental-driven interventions.

There is growing evidence to promote not only the use of kangaroo care, but also the use of music, massage, co-bedding, and Family Nurture Interventions in extremely preterm infants (288–297). Despite the use of different measures to assess stress and pronounced differences in intervention, results from these studies largely indicated improvement in HPA-axis functioning and autonomic control, as indicated by lower cortisol levels and increase autonomic stability following the intervention. These auditory and tactile interventions can be viewed as environmental enrichment, possibly stimulating cortical plasticity and attenuating the stress response in preterm infants (298). Indeed, recent studies found encouraging, but preliminary, evidence for increased microstructural maturation at term-equivalent age for preterm infants exposed to music during their NICU stay (299), as well as a greater maturation of cardiac function (296). In the long-term, both Family Integrated Care, i.e., infant care provided by families by enhancing parental support and education (300), and Family Nurture Interventions, i.e., promotes calming interaction between mother and infants, seem to have a sustained positive effect of behavioral outcome, with more robust self-regulation skills and less negative emotionality at 18–21 months and 4–5 years of age, amongst others (297, 301, 302). Further research is warranted into the exact neurobiological pathways underlying the relationship between tactile and auditory interventions and preterm infant stress-regulation.

There are several reasons to hypothesize that single-family rooms (SFR) will reduce infant and parental stress, increase parental involvement, and subsequently improve infants' outcome. Nonetheless, scientific evidence on the benefits of SFR, vs. open bay, is at this point mixed and questions whether the change from open bay to SFR is justified [for a review see (24)]. Some studies reported positive benefits of private rooms, including less physiological stress, improved neurobehavioral development, and better long-term outcomes in preterm infants (303–305). On the contrary, studies also reported potential adverse effects in relation to SFR, that is, increased maternal stress (306, 307), altered infant cerebral development, and worse neurodevelopmental outcome (308), and no effects on infant salivary cortisol reactivity (309). Together, these findings suggest that consideration of the design and environment is important for the health and well-being of preterm infants admitted to the NICU, including noise control, parent-infant closeness, and parental involvement. However, much uncertainty remains regarding the design of the NICU environment, how much or what type of sensory stimulation would optimize brain development in preterm infants. It is important to realize that both neurosensory deprivation, a consequence of SFR, and neurosensory overexposure, a consequence of open ward, might be maladaptive (310).

In sum, a range of aspects of the physical environment is pivotal for stimulating development of the preterm infant. Research showed beneficial effects of parent-infant bonding, sensory stimulation, and the use of private family-rooms on mitigating postnatal stress in preterm infants. Moreover, these interventions seem to promote infant self-regulation, by adequately utilizing their neurophysiological modulatory system to safeguard oneself from excessive over-stimulation and arousal. Hence, appropriate tactile and auditory stimulation seems sufficient to induce improvement in self-regulation. Nonetheless, results are not consistent, and the degree to which these improvements are sustained remains inconclusive. Also, several methodological challenges, such as lack of standardization, possibly introduce confounding effects.

CONCLUDING REMARKS

In summary, the reviewed literature suggests that stress has both proximate and long-lasting detrimental effects on brain and behavior in preterm-born individuals. A key question is whether there are, so-called, "sensitive" periods of pre- and post-natal development during which stress-regulatory mechanisms are established. Indeed, research indicates that not only the HPA-axis and ANS are formed during the embryonic, fetal, and postnatal period, also large-scale brain networks implicated in the central response of

BOX 1 | Unanswered questions.

1. Following premature birth, how do postnatal stressor type, timing, and duration translate to changes in neurobiological systems and subsequent outcome across the life course?
2. Which (combination of) neurobiological mechanisms underlie vulnerability or resilience following postnatal stress?
3. Are neurobiological changes following premature birth (i.e., physiological, neural network, and epigenetic) indicative of maladaptive functioning or do they also occur as a result of adaptive processes in stress-regulatory systems?
4. Can targeted and individualized neonatal interventions reverse morphological and functional remodeling of neurobiological systems and lead to improved outcomes? Also, when are these interventions able to buffer the effects of postnatal stress on neurobiological development to mitigate long-term risk for affective and social functioning?
5. What is the potential of identifying individual developmental trajectories, and the subsequent early identification of who is and who is not at risk following preterm birth and chronic stress exposure?

stress including the DMN, SN, and ECN start to develop early on. This window of increased vulnerability offers an important clue for the cellular impairment that might underlie stress-sensitivity and long-term outcome in preterm born individuals.

A few studies have emerged to investigate the effects of "early" vs. "late" stress. Postnatal work indeed demonstrated that early stress has differential and long-lasting effects on brain development, compared to stress experienced around term-equivalent age. Importantly, some studies also suggested an independent effect of postnatal stress on brain development, rather than prematurity *per se*. The current review underscores increasing evidence that postnatal stress might persistently impact later functioning in part by affecting neurobiological systems, including the HPA-axis, ANS, large-scale brain networks, and gene expression. Also, an increasing number of intervention studies found preliminary evidence for the possible beneficial effects of parent-infant bonding, sensory stimulation, and family rooms on mitigating the detrimental effects of postnatal stress. However, the reviewed evidence is far from conclusive and there is a paucity of clinical studies on the subject of prematurity and stress resilience and vulnerability. We have only started to investigate the role of postnatal stress in resilience and vulnerability to developing psychiatric disorders following prematurity. Therefore, our schematic presentations of some proposed relations (see **Box 1** and **Figure 2**) should not be considered comprehensive, rather, it should guide future research toward possible modulating factors between postnatal stress and future resilience or vulnerability in preterm born individuals.

To better understand and reduce the impact of postnatal stress and prematurity on brain development, psychopathology, and possible mechanisms, one should consider integrating several additional issues into future studies (see **Box 1** for unanswered questions). First, existing research has predominantly focused on the effects of physical stressors, such as skin-breaking procedures, rather than taking into account different stressor types, including maternal care and environmental stress. As pointed out previously, there is a great dependence of stressor type in characterizing risk factors. In other words, each stressor has distinct and possibly cumulative effects on later development of brain and behavior, and failing to account for stressor type might greatly impact results. Hence, there is a great need for systematic research on the possible detrimental effects of postnatal stress. These investigations should particularly focus on the possible long-term effects of postnatal stress on later development of brain and behavior, including adolescence and adulthood.

Second, it is important to note that only a minority of preterm born individuals will develop a psychiatric disorder. It has become clear that several neurobiological pathways might react differently to neonatal stress in resilient and vulnerable individuals. However, the specific factors that interact and account for these differences are still undetermined. Behavioral studies reported that positive parental behavior is favorable and predisposes preterm individuals to stress resilience (297, 301, 302, 311, 312). On a neurobiological level, detailed analyses of factors that account for and interact with variation in several moderating and mediating factors (e.g., HPA-axis, epigenetics), in both vulnerable and resilient individuals, is lacking. Such research would benefit from prospective longitudinal studies, with a systematic assessment of postnatal stress, and the association with changes in neurobiological systems and later affective and social functioning. For instance, as stress has a global effect on brain functioning, investigating large-scale brain networks gives a greater insight into the reorganization of connectivity patterns, rather than limiting analyses to predefined regions of the brain (313). To date, it remains unclear whether and how acute stressors relate to shifts in resource allocation between large-scale brain networks, and whether specific neural patterns might underlie resilience in preterm born individuals. Most importantly, how these networks dynamically unfold as a function of stress, and whether postnatal stress might alter these dynamics, remains unanswered.

Third, the ability to "bounce-back" is not viewed as a stable trait, rather, it is viewed as malleable and easily modified by interventions (314). Certain postnatal interventions appear to be beneficial in reducing the detrimental effects of postnatal stress, particularly interventions increasing parent-infant bonding (e.g., kangaroo care). However, considerable

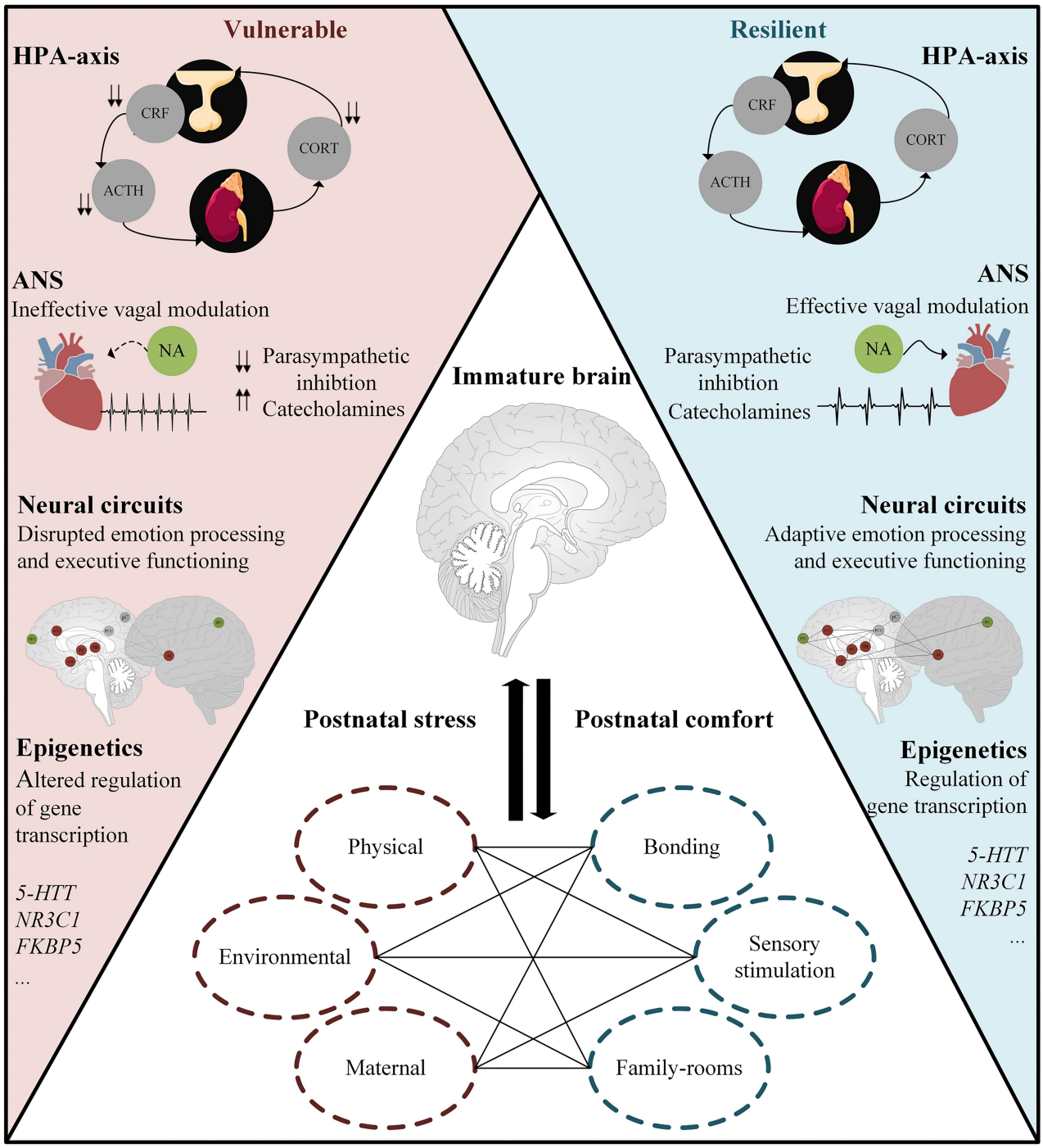

FIGURE 2 | Resilient functioning in preterm born individuals exposed to chronic postnatal stress might be facilitated by; the ability to regulate and dampen stress responsivity; effective vagal modulation; homeostasis in large-scale neural networks underlying emotion processing and executive functioning; and adaptive regulation of gene transcription. Middle panel: Postnatal factors influencing brain maturation and, in turn, the onset/development of stress-related disorders such as anxiety and depression. ACTH, adrenocorticotropic hormone; Cort, cortisol; CRF, corticotropin-releasing factor; HPA-axis, hypothalamus–pituitary–adrenal axis; ANS, autonomic nervous system; NA, Nucleus Ambiguus; *5-HTT*, serotonin transporter; *NR3C1*, glucocorticoid receptor; *FKBP5*, *FK506* binding protein 5 and acts as a co-chaperone that modulates glucocorticoid receptor activity.

uncertainty remains as to whether these beneficial effects persist over time, and what, if any, neurobiological systems are remodeled.

Preterm born individuals have an increased risk for developing psychiatric sequelae, and this heightened vulnerability might have its origin in the postnatal exposures.

Fortunately, research investigating the role of postnatal stress on later development in preterm born individuals has gathered momentum over the past two decades, and an increasing number of studies have focused on ways to diminish the detrimental effect of postnatal stress. Although the mechanisms that lead to resilient phenotypes is far from being fully determined, the current review identified several potential factors that might facilitate an adaptive stress response in the face of adversity. An increased understanding of the neurological, physiological, and epigenetic circuitry underlying resilience in preterm born individuals might be a starting point for the development of targeted and individualized intervention and prevention programs.

AUTHOR CONTRIBUTIONS

FL drafted the paper. CV, MT, and MB provided critical revisions. All authors approved the final version of the paper prior to submission.

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