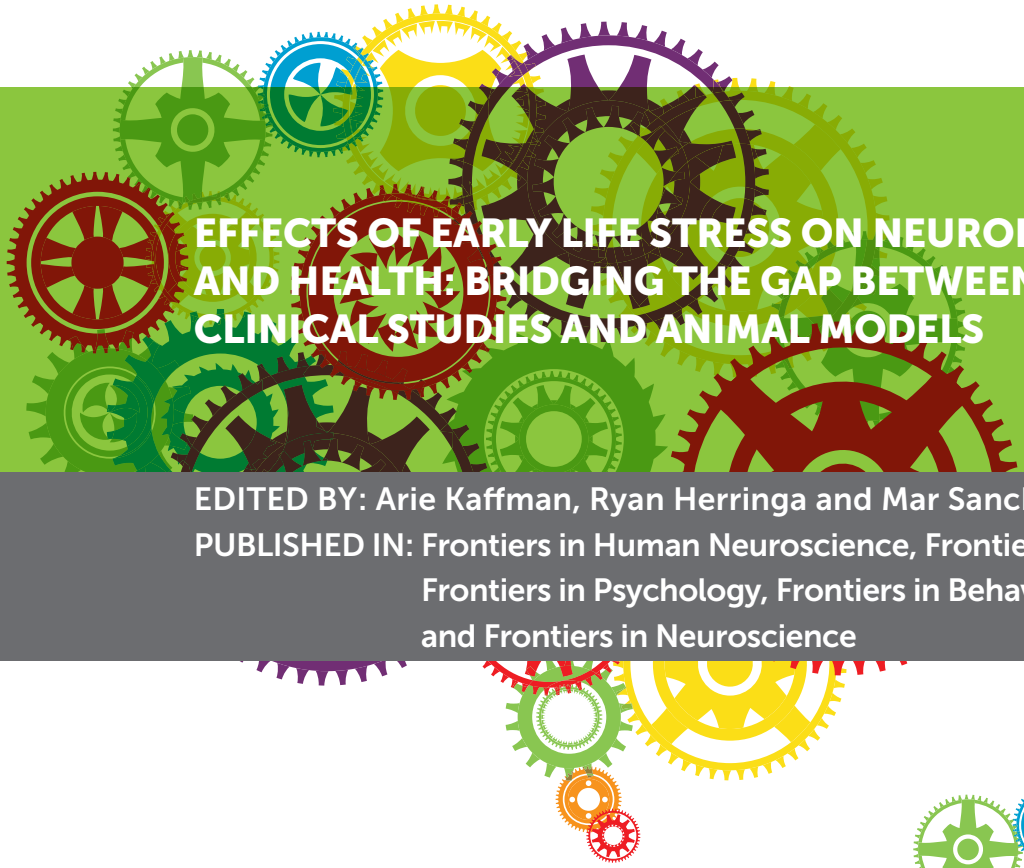

EFFECTS OF EARLY LIFE STRESS ON NEURODEVELOPMENT AND HEALTH: BRIDGING THE GAP BETWEEN HUMAN CLINICAL STUDIES AND ANIMAL MODELS

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EFFECTS OF EARLY LIFE STRESS ON NEURODEVELOPMENT AND HEALTH: BRIDGING THE GAP BETWEEN HUMAN CLINICAL STUDIES AND ANIMAL MODELS

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EDITORIAL
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Editorial: Effects of Early Life Stress on Neurodevelopment and Health: Bridging the Gap Between Human Clinical Studies and Animal Models

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Editorial on the Research Topic

Effects of Early Life Stress on Neurodevelopment and Health: Bridging the Gap Between Human Clinical Studies and Animal Models

Early life adversity (ELA) is a broad term used to describe environmental conditions that negatively impact normal development (Shonkoff et al., 2012). These include different forms of parental maltreatment/neglect, bullying, poverty, exposure to war or neighborhood crimes, discrimination, malnutrition, and environmental pollution. In many cases, these toxic environmental conditions co-occur leading to impairment in normal development and the emergence of multiple psychiatric and other medical conditions (Nemeroff, 2016; Teicher and Samson, 2016). A recent analysis estimated the economic burden of childhood maltreatment in the US alone at 2 trillion dollars per year (Peterson et al., 2018), far exceeding estimated costs of all cancers combined (Mariotto et al., 2011). Precisely how ELA alters neurodevelopment and increases the risk for such a broad range of psychiatric and medical conditions and how can these effects be reversed are difficult questions to address in humans. This is due to the heterogeneity of the adversities, differences in genetic susceptibility, and limited tools to rigorously examine and manipulate the human brain especially early in development (Smith and Pollak, 2021). Animals exposed to ELA show several structural and behavioral changes reported in humans and this preclinical work has provided some important insights into possible mechanisms and novel diagnostic and therapeutic interventions (White and Kaffman, 2019). Unfortunately, much of the clinical and preclinical efforts run in parallel tracts with little coordination and cross talk between the two approaches.

The goal of this eBook was to solicit input from researchers on both sides of the "scientific divide" on how to better coordinate the effort between clinical and preclinical/basic neuroscience and to highlight specific areas that show great promise for a productive translational collaboration and the development of new diagnostic and therapeutic tools. The response from the scientific community on how to bridge the gap between pre-clinical and clinical work was both fascinating and diverse and we conceptually grouped it into 5 themes: (1) Novel Mechanisms and Potential Therapies, (2) Insights Regarding Prenatal Stress, (3) Cumulative vs. Dimensional Models, (4) The Moderating Effects of Sex, and (5) Gene by Environment Interaction.

NOVEL MECHANISMS AND POTENTIAL THERAPIES

Several papers provided novel mechanistic insights that may lead to the development of new screening tools and interventions. Islam and Kaffman point out that ELA perturbs myelin development in humans, non-human primates and rodents. The authors review novel mechanistic insights from recent studies in rodents and discuss specific examples of how insights from animal studies coupled with the development of novel myelinating agents provide a promising new pharmacological frontier to treat cognitive and behavioral consequences of ELA in humans. Guadagno et al. review recent findings showing that some forms of adversities disrupt the development of perineural nets surrounding GABAergic neurons in rodents and humans. They propose that disruption of these structures located around neurons alters inhibitory/excitatory outputs in fronto-limbic circuits that are responsible for threat detection and anxiety. This is a novel and intriguing mechanistic animal model nicely complements Hanson and Nacewicz proposal that disruption of inhibitory/excitatory balance drives ELA mediated changes in amygdala volume (see "Cumulative vs. Dimensional Models" section below).

Coplan et al. report that the kynurenine pathway is dysregulated in the brain of adult macaque females exposed to variable foraging demand -a form of ELA- as infants, with concentrations of kynurenine metabolites in cerebrospinal fluid (CSF) serving as good markers of the different routes of the pathway. This is the first study to describe changes in kynurenine metabolites in an animal model of ELA, providing a novel mechanism and a potential therapeutic target to address neuroimmune modulation seen in some early adversities. Duffy and Roth provide a thoughtful discussion about the challenges of using peripheral epigenetic markers of DNA methylation to predict changes in the prefrontal cortex using a maternal-maltreatment rat model.

INSIGHTS REGARDING PRENATAL STRESS

Several authors focused on prenatal stress as an important area of productive translational work. Duffy et al. reviewed elegant mechanistic studies in rodents indicating that prolonged parental stress alters non-coding small RNA content in the sperm that in turn guides differences in neurodevelopment, physiology, and behavior in the offspring. These preclinical findings provide a novel conceptual framework for evaluating similar changes in non-coding small RNAs sperm content seen in stressed men. Fitzgerald et al. highlights the chronicity of prenatal stress in clinical settings as opposed to the more acute stress used in rodent models of prenatal stress. The authors explore differences in outcomes between chronic and acute prenatal stress and advocate for the inclusion of more chronic prenatal stress paradigms in rodents.

Perinatal iron deficiency is associated with abnormal neurodevelopment and is commonly seen in early adversities associated with severe poverty and famine. Using a naturalistic non-human primate model of prenatal iron deficiency, Vlasova et al. found structural MRI changes in both gray and white matter of 1 year old infants diagnosed with iron deficiency during the perinatal period (i.e., 1–2 months). Interestingly, repleting iron stores during the perinatal period did not reverse the MRI changes suggesting that earlier preemptive supplementation of iron, especially during the prenatal period, might be necessary.

Ceniceros et al. present surprising and thought-provoking results in macaques suggesting that maternal relocation during pregnancy reduced measures of anxiety in 3–4 month old offspring. These findings were also seen in infants that were cross-fostered postnatally and are consistent with the notion that mild-stressors during the prenatal period might have an inoculative/protective effect on neophobia in the offspring. Finally, Georg et al. used machine learning to identify variables that increase parental stress in response to infants with early regulatory disorder (i.e., colic). The authors suggest that interventions that reduce parental stress will likely improve long-term outcomes in infants with regulatory disorder.

CUMULATIVE VS. DIMENSIONAL MODELS

Several articles provided interesting insights into a central debate between the "cumulative risk model" in which multiple stressors are simply added to predict health outcomes (Evans et al., 2013) and the "multi-dimensional model" arguing that distinct types of adversities cause different developmental outcomes that are not necessarily additive and are better characterized using a multi-dimensional grid (Mclaughlin et al., 2014; Mclaughlin and Sheridan, 2016). Using animal and human studies, Hanson and Nacewicz propose an inverted-U-allostatic load model as a conceptual framework to explain inconsistent findings on the effects of ELA on amygdala size. Simply put, low-moderate levels of adversity lead to relative amygdala expansion whereas sustained and severe activation of the amygdala during childhood and adolescence leads to excitatory neurotoxic-mediated dendritic atrophy, possibly cell death, and a reduction in amygdala volume. The authors propose that physical or mental perception of "entrapment/inescapability" is a critical dimension driving changes in excitatory/inhibitory balance and ultimately amygdala volume.

Gotlib et al. reported that severity of ELA predicted levels of depression during the covid pandemic in adolescents. This severity effect was more pronounced in females and was mediated by perceived levels of stress. These findings highlight the role that "perceived stress" plays in mediating the risk for depression and suggest that clinical interventions that target stress perception may be particularly effective in individuals exposed to high levels of adversities. It is tempting to speculate that levels of "perceived stress" noted by Gotlib et al. is highly related to the perception of "entrapment" emphasized by Hanson and Nacewicz.

Using a rat model of ELA, Zhang et al. reports an interesting relationship between levels of BDNF in the prefrontal cortex of juvenile rats and the severity of the adversity. More specifically, juvenile rats exposed to 3 h of maternal separation had lower

BDNF in the prefrontal cortex, and had higher levels of anxiety and corticosterone compared to juvenile rats exposed to only 15 min of maternal separation. These preclinical findings raise the possibility that levels of BDNF in the prefrontal cortex may partly mediate the cumulative risk outcomes seen clinically across multiple psychiatric conditions. Orso et al. reports robust increase in anxiety-like behavior in mice exposed to a combination of two forms of early adversities (i.e., maternal separation and limited bedding and nesting). These findings underscore the need for additional preclinical work studying the effects of "simple," "multiple," and "co-occuring" stressors on neurodevelopment. Such work will better mimic clinical settings and help address the different predictions made by the cumulative and the dimensional models (White and Kaffman, 2019).

THE MODERATING EFFECTS OF SEX

The notion that males and females respond differently to ELA has received growing attention in recent years including some very interesting findings reported in this collection. For example, consistent with previous work, Stenson et al. found that ELA accelerated puberty in black girls. Using statistical mediation analysis they also showed that accelerated puberty significantly contributed to increased anxiety in black girls. ELA did not accelerate puberty in black boys and there was no significant relationship between timing of puberty and anxiety, highlighting important differences in the effects of ELA on puberty and anxiety in males and females. Raymond et al. found that women that were exposed to ELA after age 8, but not before age 8, showed increased attentional bias to threat, findings that were not seen in men. Clarifying the underlying mechanisms by which sex alters outcomes of ELA is an area of preclinical research that desperately needs additional attention due to the long standing historical bias of conducting animal studies exclusively in males (White and Kaffman, 2019; Bath, 2020).

Ultrasonic vocalizations (USVs) in rodent pups is the equivalent of an infant cry and an effective mechanism to solicit maternal care. Granata et al. showed that exposure to daily maternal separation altered USVs mainly in males, while exposure to limited bedding and nesting (a different model of ELA) impacted USVs mainly in females indicating that males and females are affected differently by different forms of adversities. An interesting follow up question is whether exposing pups to both stressors simultaneously, as was done by Orso et al. would impact both sexes and whether the magnitude of the changes would be enhanced in the presence of this complex stress.

GENE BY ENVIRONMENT INTERACTION

The moderating effects of genetic susceptibility on consequences of ELA have been re-examined in this eBook using two well characterized genes: FKBP and the serotonin transporter. The FKBP gene plays an important role in terminating glucocorticoid mediated stress response and has been previously shown to interact with ELA to alter risk of depression and PTSD (White et al., 2012; Klengel et al., 2013). Kwon et al. extended these earlier findings to show that susceptible alleles of the FKBP gene interact with ELA to modulate volumetric changes in the left orbitofrontal cortex and the left middle temporal gyrus in non-symptomatic individuals. Wood et al. examined the effects of peer-rearing and the serotonin transporter alleles on baseline and stress-induced monoamine levels in a large cohort of infant macaques. Infants that were raised with peers in the absence of a mother had different baseline and stress-induced monoamine levels compared to infants raised by their mother in a more complex and naturalistic social context. This work also provides new insights into earlier findings in humans showing that individuals the "short" allele are more sensitive to ELA compared to individuals with the "long" allele (Caspi et al., 2003).

AUTHOR CONTRIBUTIONS

AK wrote the first draft which was then edited and modified by RH and MS. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Effects of BDNF Signaling on Anxiety-Related Behavior and Spatial Memory of Adolescent Rats in Different Length of Maternal Separation

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As an adverse form of early-life stress (ELS), maternal separation (MS) can interfere with the development of cognition and behaviors of adolescent rodents. Brain-derived neurotrophic factor (BDNF) is involved in the regulation of brain development and function, but the molecular mechanisms by which BDNF regulates brain function and behavior in MS with different stressor strengths remain unclear. This descriptive study characterized the levels of BDNF in the prefrontal cortex (PFC) and plasma corticosterone (CORT) from the offspring of rats exposed to early handling (EH, 15-min separation per day) and prolonged MS (PMS, 180-min separation per day), during postnatal days (PND) 1-21. The behavioral and biochemical analyses were performed during adolescence (PND 42-56). PMS resulted in reduced weight and decreased locomotor activity in the open field test and Y-maze task compared to control (CON) group, with EH showing an intermediate phenotype. BDNF protein levels in the PFC were lower in PMS compared to EH and further reduced in CON male rats. Plasma CORT levels were higher in PMS compared to CON with EH again showing intermediate levels. Neither PMS or EH affected spatial learning in the Y-maze task. These findings indicate that longer periods of maternal separation are necessary to increase anxiety-like behavior, elevate CORT levels, and further suppress BDNF levels in the PFC, providing a possible mechanism to explain why more severe forms of ELS lead to more significant psychiatric and medical consequences later in life.

Keywords: maternal separation, anxiety-related behavior, spatial memory, brain-derived neurotrophic factor, prefrontal cortex

INTRODUCTION

Early-life stress (ELS) is a broad term used to describe various forms of stress experienced in early life, including physical, sexual, and emotional forms of abuse and neglect suffered by developing children (1), which can alter the normal pattern of brain development, resulting in various short- and long-term dysfunctions in cognitive, emotional, and other behavioral processes (2). Adolescence is a life period in which neurodevelopment is particularly susceptible to social influence. It is thought that early disruptions of social interaction in young children may affect brain development and have long-term neurochemical, endocrine, and behavioral effects (3). Clinical research has found an increased risk for developing depression, post-traumatic stress disorder, and schizophrenia among victims of childhood abuse and neglect (4). More than 50% of children worldwide are estimated to have been exposed to early stress (5), and these children are more likely to suffer from psychological health problem, especially with severe cognitive impairment (6), within a few months or a few years in the absence of a good environment or family support.

Maternal separation (MS) is a rodent model of early adversity in which pups are repeatedly separated from the mother for different periods of times (7, 8). Current evidence indicates that MS leads to some behavioral and neurochemical disorders, such as emotional behavioral deficits, increased locomotor activity, disturbance in serotonergic and dopaminergic activities, and also lead to persistent changes in brain structural plasticity (9). Other studies have shown that repeated MS leads to memory and learning deficits in experiments using the Morris water maze and object recognition tests (10). However, it has also been reported that MS can improve reverse learning in adolescent rats (8). These seemingly conflicting results may reflect experimental differences in the MS procedure, the developmental age at which the testing was performed, or the cognitive test used (11).

Due to the variability of the MS models, the same anxiety and cognitive behavior changes cannot always be found in the early stressed population. Moreover, some studies have reported that mild separation stimuli may enhance the ability to withstand stress in adulthood. The central argument of such studies was based on the premise that exposure to new stimuli during infancy makes animals more resilient to stress (12). In contrast, Lupien found that depriving pups of prolonged maternal contact during postnatal development did not attenuate the stress-induced response, but rather negatively affected their ability to manage future stressors (13).

Clinical data and meta-analysis have confirmed the deleterious effects of adversity such as physical, sexual, and emotional abuse (14), death or separation of parents (15), out of home care for children on the risk of mental illness (16). Importantly, experiencing more or longer periods of early adversity increased the risk of disease, leading to more serious adverse outcomes. Research has shown that the risk increased by approximately 50% in individuals who have experienced one adversity, and by nearly 300% among individuals with four or more adversities (16, 17).

To elucidate the causal effects of early life experiences, rodents have been widely used as models for studying the disruptions of maternal offspring relationships (18). Previous research used protocols that included two postnatal procedures during the postnatal day (PND) 1-21, namely early handling (EH), which consisted of daily separations of the offspring from their mother for 3-15 min, and prolonged MS (PMS), which consisted of daily separation for 180 min (19). EH has been reported to have strong effects on the behavior of young adult rats, including a reduction in fear-related behaviors in spontaneous and conditioned tasks (20). The long-term effects of PMS procedures in early adulthood are the opposite of EH, including the increase of fear avoidance behaviors and the hypothalamic-pituitary-adrenal (HPA) axis responses (18, 20). Although it is recognized that exposure to MS as a stressor can seriously affect the balance of neural structure and function in the brain, the mechanisms underlying stress in early life are still not fully understood.

Brain-derived neurotrophic factor (BDNF), which is one of several endogenous proteins, plays a key role in the survival and development of the brain and peripheral neurons (21). A common genetic variation in the BDNF gene, the Val66Met, is associated with specific mood, cognitive and neuroanatomical alterations in disease (22). Research has suggested that BDNF Val66Met carriers with high levels of childhood abuse had more severe cognitive deficits than normal controls (23). In addition, BDNF can directly influence the HPA axis modulation by altering the expression levels of corticotropin releasing hormone, thereby affecting the evaluation and memory retrieval of novel information in the brain, and regulating the behavioral coping responses (24, 25). Previous studies have demonstrated that single or repeated MS caused a significant reduction in the expression of the neurotrophin BDNF in adulthood (26). However, previous studies did not find significant changes in the MS-induced alterations in BDNF in the prefrontal cortex (PFC) (27). These studies reported inconsistent behavioral and neurochemical effects and the mechanism by which MS affects cognitive functions in rodent models and what these cognitive changes are in different postnatal manipulations remain largely unknown.

In this study, two types of stress models (EH and PMS) were used to elucidate their effects on the behavior and endocrine function of offspring, as well as the relationship between BDNF expression in PFC and anxiety and spatial memory behavior in adolescent rats that had experienced early stress. We hypothesize that more severe forms of ELS (e.g. PMS) cause more severe suppression of BDNF compared to EH and control groups, and the corticosterone (CORT) levels may play a key role in this.

MATERIALS AND METHODS

Animals

Maternal Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of Weifang Medical University (Weifang, China). Prior to the experiment, a simple field experiment was performed to screen the female rats for mating. The inactive, stressed or anxious rats were screened out by observing the extent of spontaneous activity to avoid

influencing the experimental results. Animal experiments and handling were conducted in accordance with the National Institutes of Health guidelines (Use of Laboratory Animals) and were approved by the Weifang Medical University Animal Care and Use Committees. Upon arrival, the SD rats were allowed to acclimatize for 1 week, prior to impregnation, with food and water *ad libitum*. Maternal SD rats conceived pups in their natural state without external stimulation during pregnancy. The rats were housed in temperature-controlled (23 ± 2°C) and humidity-controlled (50 ± 5%) rooms, and maintained on a 12-h light/12-h dark cycle (lights on from 07:00 to 19:00) with food and water *ad libitum*. In order to minimize the potential confounding effect of litter (28), each experimental group consisted of rats derived from at least three different litters, and 3-5 pups in each litter. Neonatal SD rats and their male offspring were used in this study. On PND 21, the male animals were weaned and housed in same-sex groups.

Maternal Separation (MS) Procedure

Male offspring were randomly assigned to the EH group (MS for 15 min daily; 08:00-08:15 a.m.; n=11 L), the PMS group (MS for 180 min daily; 08:00-11:00 a.m.; n=11 L), and the control (CON) group (no separation; n=11 L) from PND 1-21 (29, 30). During the separation process, the mother was transferred into a holding cage placed in an adjacent room to minimize olfactory and auditory communication with the pups. During this period, the pups from both EH and PMS conditions were removed from the home cage and kept in an incubator at 30 °C. At the end of the separation, the pups and the mothers were returned to their maternity cages. The control subjects were exposed to the normal husbandry procedures in the animal facility.

Body Weight Gain

The body weight of the offspring was recorded weekly, during PND 7-56, to ensure that the body weight was not a confounding factor and did not affect the behavioral results.

Open Field Test

The OFT is typically performed to measure the locomotor activity and anxiety-like behaviors in rodents (31). The selected rats from each group were evaluated by the OFT during their dark cycle (between 01:00 and 05:00 p.m.) on PND 42. The apparatus used consisted of a square box (80 cm × 80 cm × 50 cm), with a field that was divided into 25 squares using the Smart-3.0 video tracking system (Panlab S.L., Barcelona, Spain). During the test, each rat was placed in the center of the open field and allowed to explore the arena freely for 5 min. The number of crossings (all four limbs), upright, carding, fecal grains, and time spent in the central area of the apparatus were observed. The apparatus was cleaned with 70% ethanol between each test to eliminate any olfactory cues left by the previously rat.

Y-Maze Test

Cognitive and motor performances of the offspring of rats were assessed in a Y-maze, as it is strongly associated with the PFC (32, 33). The Y-maze apparatus consisted of three arms made of black opaque metal plates. Each arm was 50 cm long, 30 cm high, 18 cm wide, and at an angle of 120 degrees with the other two arms. Rats were habituated to the Y-maze for 3 days (between 08:00 a.m. and 11:00 a.m., 5 min explorations per day) from PND 42 onwards. Following habituation, the rats were trained to perform the task, where one branch arm was blocked to force the animal to enter the open branch arm. Then the animal received one food pellet (only one food was given for each training), and they were free to explore in the maze for 10 min (training trial). The mice were then returned to their cages. One hour later, the rats were reintroduced into the starting arms and allowed to freely explore all three arms for 5 min (test trial). The following parameters were measured: total number of arm entries in the maze and percent novel arm entry alternation (number of alternations/number of arm entries- 2).

Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

To determine the influence of MS on the blood CORT levels, rats were euthanized with 8% chloral hydrate (400 mg/kg, I.P.). Heart blood samples were collected in the morning (between 08:00 and 09:00 a.m., time of secretion peak) for the ELISA analysis on PND 57. The heart blood samples were drawn into EDTA-coated tubes, maintained on ice, centrifuged at 4,000 g for 10 min, and then the plasma was collected. The CORT concentration was measured using a rat corticosterone, CORT, ELISA kit (AD3193Ra; Andygene Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's protocol. The samples (or standard) and the conjugate were added to each well and the plate was incubated for 1 h at room temperature without blocking. The wells were washed several times with various buffers and the proper color developed. The plasma CORT levels were detected by absorbance of samples at 405 nm, which were compared to known CORT standards using an automated Epoch microplate spectrometer and the KinetiCalc Jr. Software (Bio-Tek Instruments, Winooski, VT, USA).

Immunohistochemistry Analysis

The immunohistochemistry analysis was performed in accordance to a previous report (34). Three rats in each group were euthanized and transcardially perfused (0.01 M cold phosphate-buffered saline (PBS) for 5 min followed by 4% cold paraformaldehyde for 20 min). The brain of each rat was then stored at 4°C in 4% paraformaldehyde for the immunohistochemistry analysis. The PFC of the brain was transferred into 30% sucrose in 0.1 M cold PBS (4°C) until it sank. Coronal slices (20 μm thick) from the bregma (2.00 to 4.00 mm) were sectioned as the PFC and collected on the poly-lysine-coated slides. Each section was categorized anatomically according to the distance from the Bregman by using the rat brain atlas (35). The sections were dried for 2 h at 60°C and then treated with 0.3% H_2O_2 in methanol for 15 min to inactivate nonspecific peroxidase reactions. The sections were rinsed three times with PBS for 15 min and then the sections were blocked with 4% normal goat plasma at room temperature for 30 min. The primary antibody against BDNF (Anti-BDNF, 1:500, ab108319; Abcam, Cambridge, UK) was diluted with primary antibody dilution buffer and incubated at 4°C overnight. After repeated washing, the sections were incubated with the secondary antibody (Horseradish peroxidase conjugated affinity purified goat anti-

rabbit IgG (H + L), 1:200 dilution, ZB-2301; ZSGB-Bio Co., Beijing, China) for 20 min at room temperature. The samples were rinsed and incubated for an additional 30 min to react the samples with a DAB (3,3'-diaminobenzidine) kit (ZLI-0931; ZSGB-Bio Co.) for color reactions at room temperature. The sections were counterstained with hematoxylin for 3 min, rinsed, dehydrated, cleared in xylene, and cover-slipped. The sections were subsequently examined on an Olympus FluoView 1200 confocal microscope system (Olympus Corp., Tokyo, Japan), and three images of representative PFC areas (400 × and 200 ×) were acquired per animal and the numbers obtained in each image were averaged. The number of positive cells was calculated using ImageJ software.

Western Blot Analysis

The Western blot analysis was performed in accordance with a previous publication (36). The PFC was collected and homogenized on ice (n= 3). The protein of the PFC was extracted with a RIPA buffer (P0013B; Beyotime, Shanghai, China) containing the protease inhibitor cocktail (ST506, Beyotime) on ice. The bicinchoninic acid (BCA) assay was used to determine the protein concentration. Equal amounts of protein samples (30 μg) were denatured by heating at 95°C for 10 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% SDS-PAGE gel (ran for 1.5 h at 120 V). Then, separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot wet transfer system (transferred for 2 h at 110 mA) with a transfer buffer for Western blot analysis. After transfer to the membrane, the PVDF membrane was blocked with 5% non-fat dry milk, in 0.05% Tween-20 in PBS (PBST). Subsequently, the membrane was incubated overnight at 4°C with the primary antibody (anti-BDNF, 1:1000, ab108319, Abcam). The following day, the membranes were subjected to three sequential 5 min washes with PBST, washed three times for 10 min with PBST, and then incubated for 1 h at room temperature with the secondary antibodies (Horseradish peroxidase-conjugated affinity purified goat anti-rabbit IgG (H + L), 1:200 dilution, ZB-2301, ZSGB-Bio Co.). The protein bands were visualized in an electrochemiluminescence (ECL) solution (WBKLS0050; Millipore, Billerica, MA, USA) for 1 min and the gray value of the immunoreactive protein band was quantified with the Image J software (NIH, Bethesda, MD, USA). An overview of the experimental procedure is depicted in **Figure 1**.

Statistical Analysis

All data were expressed as the mean ± standard error of mean (SEM) for the groups. The differences among groups in behavioral and biochemical results were analyzed with one-way analysis of variance (ANOVA) using the SPSS statistical software (Version 22.0, IBM Corp., Armonk, NY, USA), followed by a Fisher least significant difference (LSD) *post hoc* test. The body weight was analyzed by repeated-measures ANOVA. Pearson's correlation coefficient r was calculated to determine the specific relationship between the BDNF levels in the PFC, plasma CORT concentration and the behaviors of rats, respectively. In addition, the Kolmogorov-Smirnov test and Levene's test were used to evaluate the normality and variance homogeneity of the data distribution. The differences were considered statistically significant when $p<0.05$.

RESULTS

Effects of Exposure to Different Postnatal Manipulations on the Body Weight of Pups

The average body weight increase of the offspring of each group from PND 7-56 is shown in **Figure 2**. During PND 7-21, the mean pup weight of PMS and EH litters was similar. Furthermore, in several time points during PND 21-56 [PND 21: $F(2, 30)= 11.526$, $p= 0.000$; PND 28: $F(2, 30)= 53.096$, $p= 0.000$; PND 35: $F(2, 30)= 4.206$, $p= 0.025$; PND 42: $F(2, 30)= 6.926$, $p= 0.003$; PND 49: $F(2, 30)= 5.480$, $p= 0.009$; PND 56: $F(2, 30)= 5.887$, $p= 0.007$], there were significant differences in body weight among the groups. ANOVA results showed that age [$F(7, 24)= 831.588$, $p= 0.000$] and PMS [$F(14, 50)= 4.714$, $p= 0.000$] had a significant effect on the body weight of rats, and there was an interaction between the two treatment factors. Further

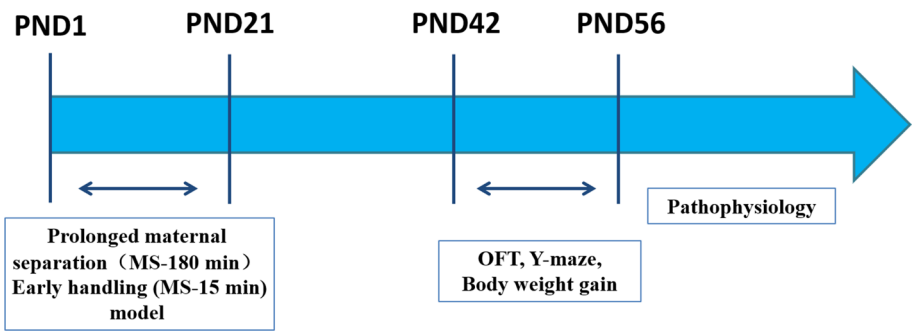

FIGURE 1 | Experimental outline. Male offspring rats were studied in parallel throughout the experiment. Two postnatal manipulations were used. PMS (MS-180): Prolonged maternal separation (MS) consists of daily separation of offspring from their mother for 180 min PND 1-21. EH (MS-15): Early handling daily 15-min separation of offspring from their mother on PND 1-21. Pathophysiology (ELISA analysis, immunohistochemistry analysis, Western blot analysis). PND: postnatal day. OFT: Open-field Test on PND 42. Y-maze on PND 42-44. Body weight gain on PND 7-56.

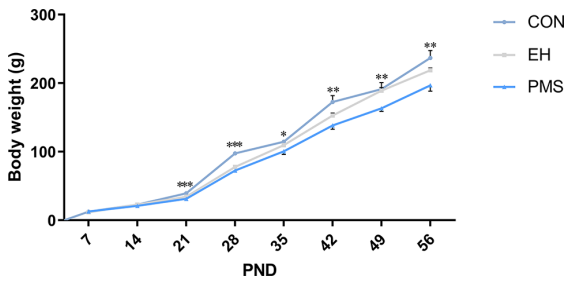

FIGURE 2 | Body weight of offspring rats during experimental procedure. Data are presented as the mean body weight of the pup in each stage. Body weight differences in the control group and EH group and PMS group (CON, EH, PMS). Points represent mean ± SEM, (n=11 rats per group). *p < 0.05, **p < 0.01, ***p < 0.001.

analysis showed that the pups in the PMS and EH groups grew more slowly than the pups in the CON group. Moreover, on PND 28 and PND 49, the weight of rats in the PMS group decreased significantly compared with that in the EH group (PND 28: p= 0.047; PND 49: p= 0.010).

Effects of Exposure to Different Postnatal Manipulations on the Spontaneous Activity of Pups

The OFT results revealed significant differences in the number of crossings [F(2, 30)= 3.425, p= 0.046, **Figure 3A**], carding [F(2, 30)= 3.922, p= 0.031, **Figure 3C**], fecal grains [F(2, 30)= 3.771, p= 0.035, **Figure 3D**] and the time spent in the central area [F(2, 30)= 18.613, p= 0.000, **Figure 3E**] among the three groups of rats, but there was no significant difference in the number of upright [F(2, 30)= 0.653, p= 0.528, **Figure 3B**]. Further data analysis revealed that reduction of the number of crossings (p= 0.016), carding (p= 0.009), and fecal grains (p= 0.012) was observed in the PMS group of rats compared to control rats. Additionally, the time in the central area of the rats was also significantly reduced (p= 0.000), but there was no significant difference between the EH group and the CON group. The tracking images were shown in the figure (**Figure 3F**).

Effects of Different Postnatal Manipulations on the Cognitive Behavior of Pups

As shown in **Figure 4**, the motor performance values (arm entries) showed significant differences among the groups [F(2, 30)= 6.134, p= 0.006], although the cognitive performances values (alternation %) did not show significant differences [F(2, 30)= 0.716, p= 0.497]. The number of arm entries in the PMS group was significantly lower than that in the control rats (p= 0.001). Exposure to EH (EH group) had no significant effect on the measured parameters. The EH and the PMS groups did not have any significant effects on the alternation %. The distance traveled indicated the differences in the space exploration between the two separation models (EH and PMS) and the control rats (**Figure 4C**).

Effects of Exposure to Different Postnatal Manipulations on the CORT Levels in the Plasma of Pups

The effects of exposure to EH (EH group) and PMS (PMS group) on the plasma CORT levels were determined as shown in **Figure 5**. There were significant differences in the CORT expression levels among the three groups [F(2, 30)= 439.177, p= 0.000]. Compared with the levels of CORT in the control group, the CORT contents were significantly increased in the PMS group (p= 0.000) and the EH group (p= 0.000).

Effects of Exposure to Different Postnatal Manipulations on the Expression Levels of BDNF Protein in the PFC of Pups

Immunohistochemistry Analysis

The number of BDNF-positive cells in the CON, EH, and PMS groups is shown in **Figure 6**. The expression of BDNF positive cells showed significant differences among the three groups [F(2, 6)= 5.362, p= 0.046] (**Figures 6A–C**). Compared with the CON group, the estimated total number of BDNF-positive cells in the PFC was significantly lower (p= 0.017) in the PMS group (**Figure 6D**), but there was no significant effect on the EH group within the mean number of the BDNF-positive cells.

Western Blot Assays

The BDNF protein levels were determined by measuring the protein bands expressed in the PFC (**Figure 6E**). The BDNF protein expression levels showed significant differences among the three groups [F(2, 6)= 10.134, p= 0.012]. Further analysis by LSD showed that the optical density of BDNF in the PFC of the PMS group was significantly lower than that in the CON group (p= 0.004). Although the average density of the BDNF protein in the EH group was also decreased, there was no significant difference between the EH group and CON group (p= 0.037).

Correlation Between BDNF Levels in the PFC, Plasma CORT Concentration and Behavioral Activity in Rats

The results of correlation analysis (**Figure 7**) showed that the plasma CORT levels were negatively correlated with time spent in the central area (OFT; r= -0.740, p= 0.000) and number of arm entries (Y-maze; r= -0.502, p= 0.003) of rats respectively, but not with the alternation % (Y-maze; r = -0.197, P = 0.273). In contrary, BDNF levels in the PFC exhibited positive correlation with time spent in the central area (r= 0.700, p= 0.000) and number of arm entries (r= 0.525, p= 0.002), but no correlation with alternation % (r= 0.246, p= 0.168). There was also a significant negative correlation between plasma CORT concentration and BDNF levels in the PFC (r = -0.915, P = 0.000).

DISCUSSION

The aim of the present study was to examine the consequences of two models of ELS that differ in terms of their severity on

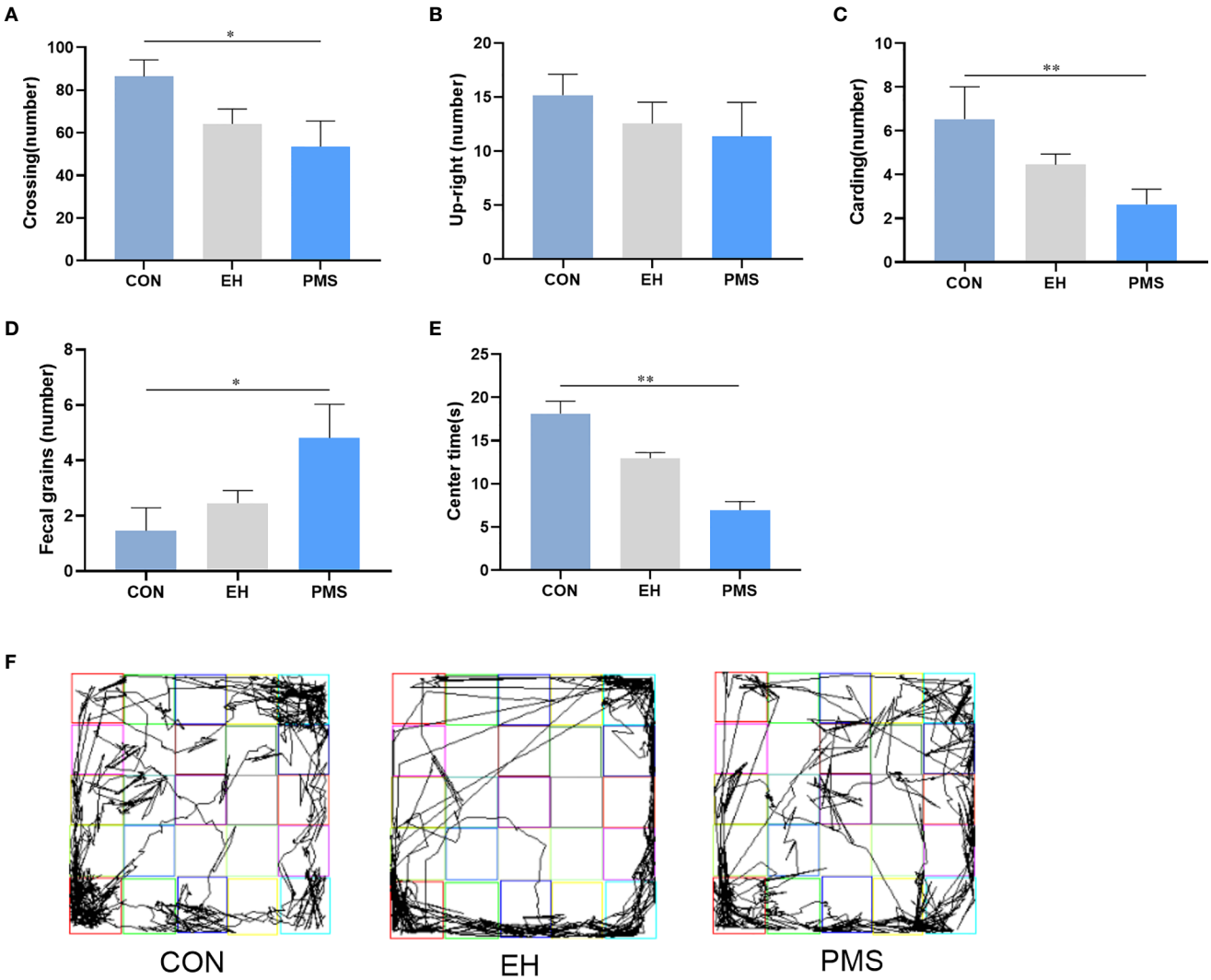

FIGURE 3 | In the open field, PMS group had reduced number in upright, carding and fecal grains compared with other groups**. (A)**: the number of crossings in the OFT. **(B):** the number of upright in the OFT. **(C):** the number of carding in the OFT. **(D):** the number of fecal gains in the OFT. **(E):** the time at the center state in the OFT. Values are expressed as the mean ± SEM (n= 11 rats per group). Representative diagrams of the pathways in the OFT from each group, and **(F)** showed the rats' mobile trajectory Figs in CON, EH and PMS group. *$p < 0.05$, **$p < 0.01$.

anxiety-like behavior and spatial memory as well as the BDNF levels in the PFC and plasma CORT levels. The results indicated the variations in postnatal separation not only influenced the development of spontaneous activity and endocrine responses to stress in the offspring but also decreased the expression levels of BDNF in the PFC.

The results of the present study found a significant increase in anxiety-like behaviors in rats exposed to PMS but not in offspring exposed to EH. This finding was consistent with a recent meta-analysis showing increased anxiety in rats exposed to PMS, that was more pronounced in younger animals (37). Further, these outcomes supported the notion that more severe forms of stress early in life increased the risk of several behavioral abnormalities, including anxiety, later in life. The lack in anxiety-like behavior in offspring exposed to EH was similar to findings reported by Eklund (38). However, others have shown that brief postpartum manipulations attenuates fear and anxiety responses (39, 40). This discrepancy was likely due to differences in methodology related to the handling procedure and the sensitivity of anxiety-like behavior to external cues such as lighting conditions, olfactory cues, time of testing, and sex of the tester (41–44).

Notably, these anxiety-related mood changes may affect the growth and development of the pups and their weight gain (45, 46). Contradictory findings have been reported about the effect that exposure to MS has on body weight, certain studies found that the body weight in pups was lower in adulthood, while other studies found no differences between stressed and control

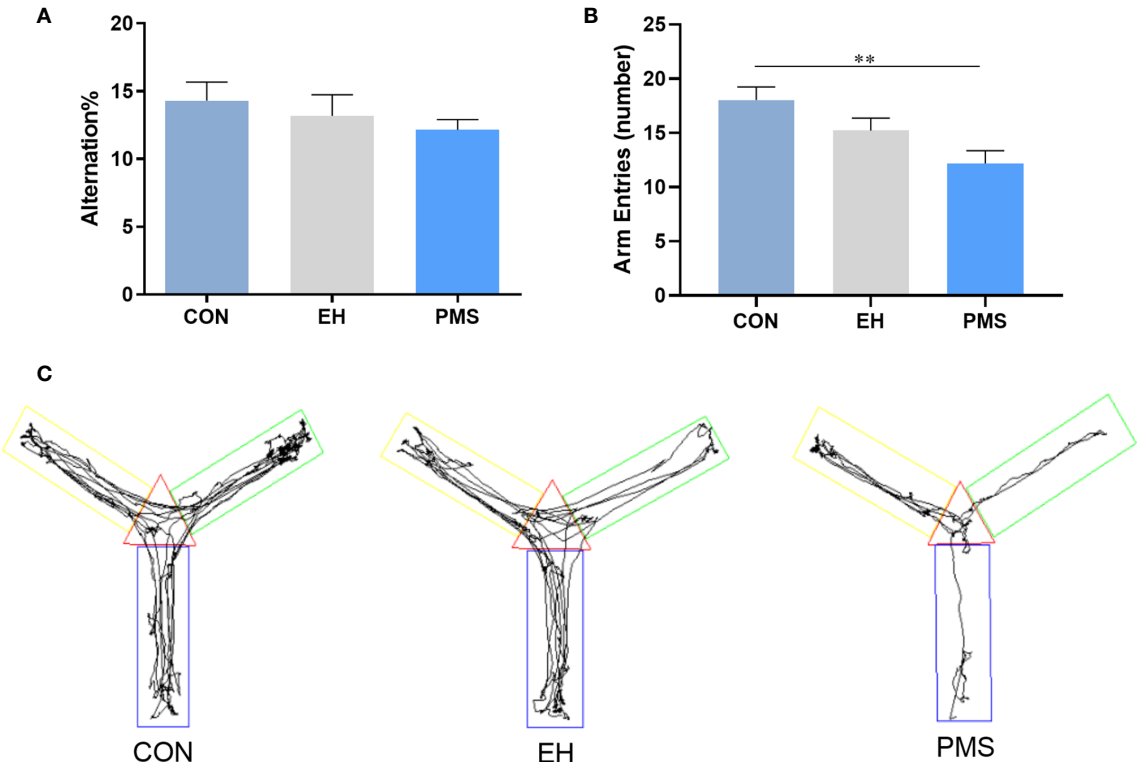

FIGURE 4 | Comparative effects of two different separation modes in the PMS and EH group on spontaneous Y-maze alternation performance of rats [**(A)**: alternation %, **(B)**: number of arm entries]. Each bar represents the mean ± SEM (n= 11 rats per group). Representative diagrams of the pathways in the Y-maze test from each group, **(C)** showed the rats' mobile trajectory Figs in CON, EH and PMS group. **p < 0.01.

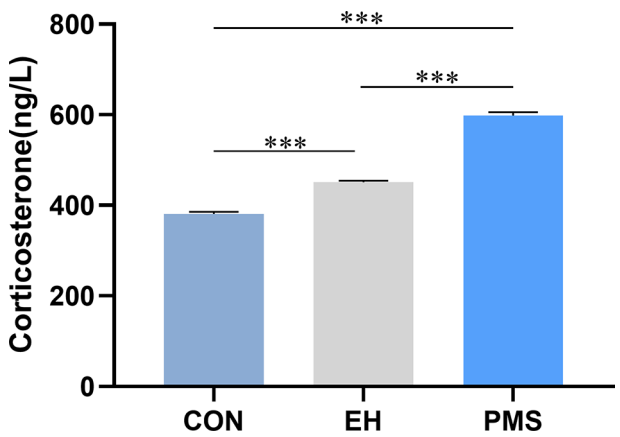

FIGURE 5 | Corticosterone (CORT) in puberty. Levels of circulating plasma CORT were assessed in the control group, EH group and PMS group (CON, EH, PMS) on PND 56. Values are expressed as the mean ± SEM (n= 11 rats per group). ***p < 0.001.

animals (47). Although the reasons behind these discrepancies remain unclear, the differences in stimulation intensity and neuroregulatory function could play significant roles in the outcomes (48). The present study found no significant difference in the average weight of pups among the three groups at PND 7 and PND 21. This finding might indicate that the food intake and weight gain of the offspring are not affected during the MS procedure as long as the pups are returned to feeding after separation. During the PND 21-56 period, the body weight of the PMS group animals was significantly different from that of the CON group rats, but there was no significant difference between the EH group rats and the CON group rats. Additionally, this study found that EH rats had lower body weight during the PND 21-28 period, although the body weight difference between the EH and CON groups disappeared in adolescence. These results indicated that PMS caused a more significant reduction in body weight compared to pups exposed to EH and was consistent with clinical findings showing that severe forms of adversity lead to stunted growth (49). A possible mechanism for the effects of PMS on weight and anxiety might be due to higher basal levels of CORT found in PMS animals. This assertion was supported by our finding that CORT levels were highly correlated with anxiety-like behavior (**Figure 7A**).

Studies using experimental models of early stress have also shown that the imbalance of stress hormones can lead to cognitive impairment (50). Adolescence is a period of extensive brain maturation in which synaptic balance and stability are developed and cognitive function and synaptic plasticity are

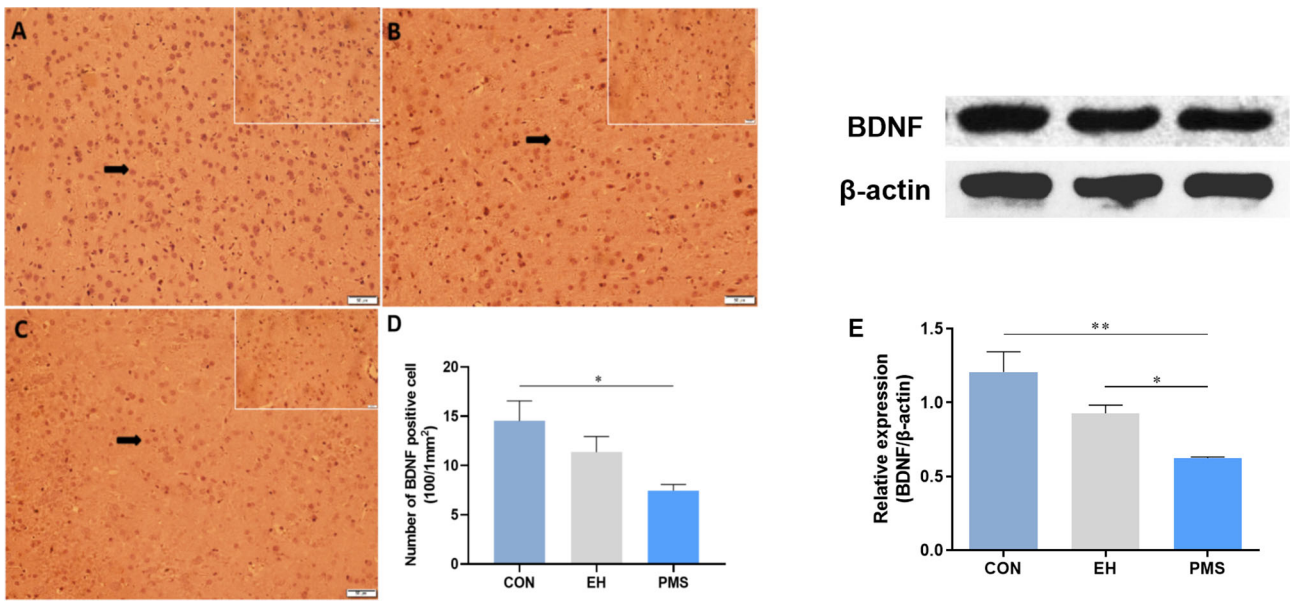

FIGURE 6 | Effects of the PMS and EH procedures on the levels of BDNF protein in the PFC. **(A)**: The figure displays BDNF immunostaining in the PFC from the CON group. **(B)**: A section of the PFC of the EH group showed the presence of BDNF positively stained cells in the outer layer of the PFC. **(C)**: The section from rats of the PMS group showed more BDNF positively stained cells in the outer layer of the PFC. Magnification 200× for **(A), (B),** and **(C)**, and 400× for the inserts (arrow). **(D)**: Quantification of the difference in BDNF positive cells in the PFC between groups. **(E)**: The Western blot analysis results of BDNF in the PFC samples from the EH, PMS and control (CON) groups. A sample of 30 μg of cellular protein from different rat brain regions was processed for Western blot assays using antibodies against BDNF and β-actin. The Intensity of the immunoreactive protein was measured using the Image J software. Values are expressed as the mean ± SEM (n= 3 rats per group). *$p < 0.05$, **$p < 0.01$.

matured (51). PFC is one of the key brain areas involved in cognitive and emotional function regulation. The synaptic plasticity of this region is very important for learning and memory processes and highly regulated by early environmental factors. Moreover, disturbances in the maturation of the PFC have been linked to the emergence of multiple mental illnesses during adolescence (52). It has been reported that ELS can interfere with the maturation process of PFC, damage the structure and function of prefrontal neurons, which is manifested in the decreased number of neurons and synaptic transmission efficiency (53). However, little is currently known about the effects of EH and PMS on PFC development and function. Although, EH and PMS did not affect alternation % in the Y-maze, there was a significant reduction in BDNF levels in the PFC of PMS rats compared to EH and CON (**Figure 6**). This was important because BDNF mediates long-term potentiation (a form of synaptic plasticity) in the PFC and was considered as the key biological mechanism underlying associative learning and memory recognition (54). Genetic analysis revealed that Val66Met, the BDNF allele, was associated with a high risk of suicide ideation, emotional vulnerability to stressful life (55), and prefrontal cortex atrophy (56). Similar behaviors were found in BDNF +/met knock-in mice that exhibited depression- and anxiety-like behaviors, and impaired working memory after stress exposure (57). This polymorphism seemed to also predict the stress response and remission incidence (58). In addition, high concentration CORT have been shown to inhibit the secretion of BDNF in the hippocampus (59). This finding was consistent with our findings that CORT levels were negatively correlated with BDNF expression in the PFC (**Figure 7G**). Interestingly, BDNF levels were positively correlated with the time spent in the center of the open field (**Figure 7D**) raising the possibility that levels of BDNF in the PFC may play a role in programming anxiety-like behavior in juvenile male rats. Additional studies are needed to clarify whether similar changes in BDNF levels in the PFC and anxiety are also seen in female rats exposed to PMS.

Despite the large variation in ELS models, species, test age, and outcome parameters, most of the studies only reported slight behavior changes in females after experiencing early life adversity (60). According to the literature, ELS only affected the survival levels of adult-born neurons in males associated with a more seriously damaged cognitive performance in adult ELS-male rodents compared with females (61). However, we found that male rats with early stress, as revealed in the Y-maze test, showed no significant change in spatial cognitive behavior, even if the alternation rate decreased, which may only indicate the risk of potential cognitive impairment, thus requiring more cognitive behavioral tasks to advance future research on rodents of different ages and genders. In addition, it has been found that the behavior and neural outcomes of individuals exposed to ELS have changed, and that these outcomes can be passed on to their

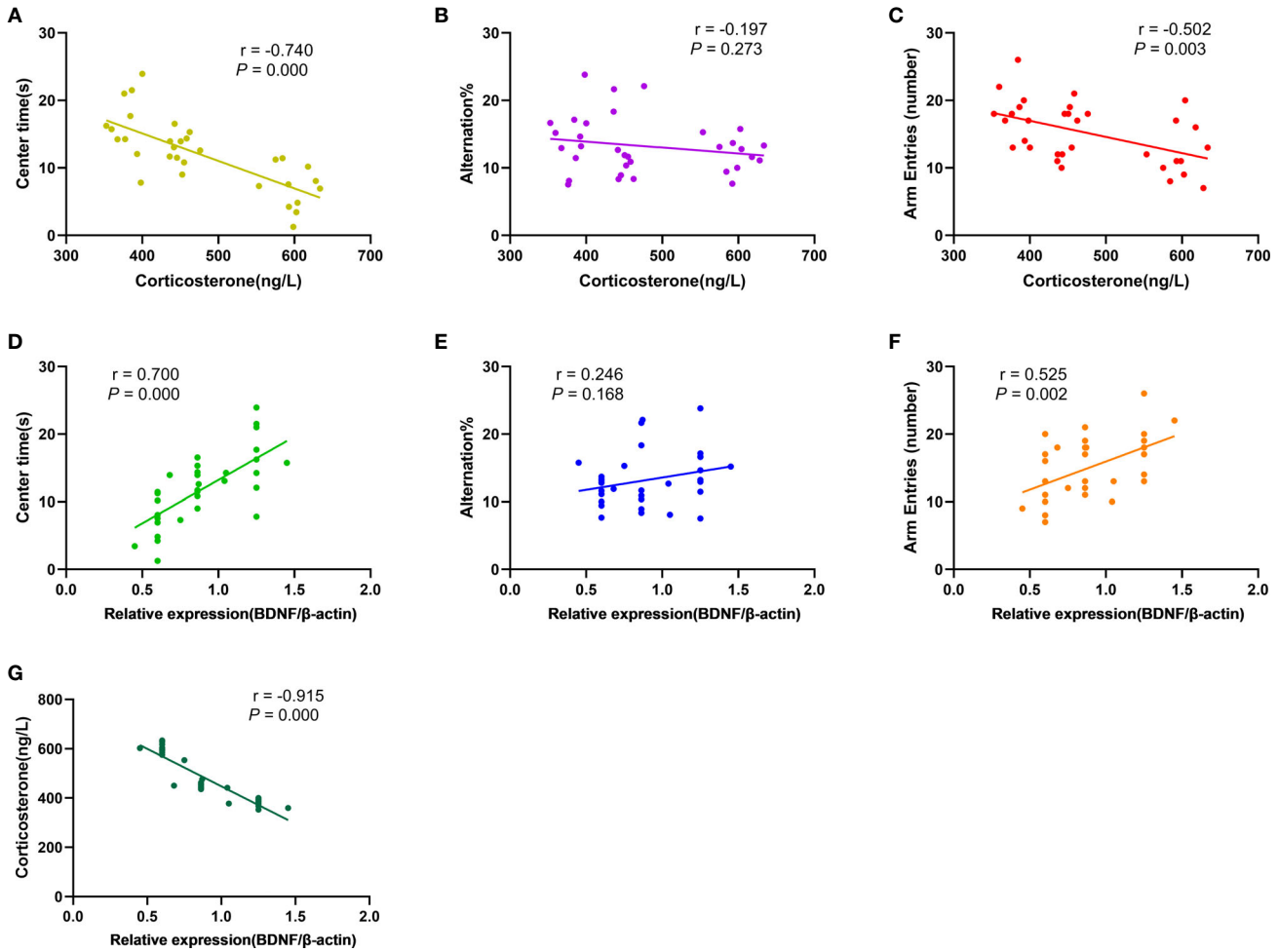

FIGURE 7 | Results of correlation analysis. **(A)**: The correlation between plasma CORT levels and time spent in the central area of rats in OFT (r= -0.740, p= 0.000); **(B)**: The correlation between plasma CORT levels and alternation % in Y-maze test (r= -0.197, P= 0.273); **(C)**: The correlation between plasma CORT levels and number of arm entries of rats in Y-maze test (r= -0.502, p= 0.003); **(D)**: The correlation between BDNF protein expression in the PFC and time spent in the central area of rats in OFT (r= 0.700, p= 0.000); **(E)**: The correlation between BDNF protein expression in the PFC and alternation % in Y-maze test (r= 0.246, p= 0.168); **(F)**: The correlation between BDNF protein expression in the PFC and number of arm entries of rats in Y-maze test (r= 0.525, p= 0.002); **(G):** The correlation between BDNF protein expression in the PFC and plasma CORT levels (r= -0.915, P= 0.000).

offspring (62). Therefore, it will be worthwhile to explore whether female rats exposed to ELS can transmit stressed phenotypes across generations as well as its potential epigenetic mechanisms.

CONCLUSION

In summary, this study demonstrated that PMS increased CORT secretion, down-regulated BDNF expression in the PFC, and induced anxiety-like behavior in adolescent pups. Exposure to brief separation (EH) did not alter anxiety-like behavior, or BDNF levels, but increased CORT levels compared to CON, but to significantly lower levels compared to PMS. These findings are consistent with a large body of clinical work suggesting that more severe forms of ELS cause more significant behavioral and physiological abnormalities. Further, our findings suggest a possible role for elevated CORT in programming BDNF levels and abnormal PFC function in more severe forms of ELS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Weifang Medical University Animal Care and Use Committees.

AUTHOR CONTRIBUTIONS

LS, XZ, and HL are responsible for the conception and design of the work. HaS, YJ, XS, GZ, and ZD are responsible for the acquisition and analysis of data for the work. QL is responsible for drafting the work for important content. HoS and YK participated in the revision of the article. All authors contributed to the article and approved the submitted version.

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Maternal Separation Combined With Limited Bedding Increases Anxiety-Like Behavior and Alters Hypothalamic-Pituitary-Adrenal Axis Function of Male BALB/cJ Mice

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Early life stress (ELS) is considered a risk factor for the development of psychiatric conditions, including depression and anxiety disorder. Individuals that live in adverse environments are usually exposed to multiple stressors simultaneously, such as maternal neglect, maltreatment, and limited resources. Nevertheless, most pre-clinical ELS models are designed to explore the impact of these events separately. For this reason, this study aims to investigate the effects of a combined model of ELS on anxiety-like behavior and hypothalamic-pituitary-adrenal (HPA) axis related targets. From PND 2 to PND 15 BALB/cJ mice were exposed simultaneously to maternal separation (MS; 3 h per day) and limited bedding (LB; ELS group) or left undisturbed (CT group). Maternal behavior was recorded in intercalated days, from PND 1 to PND 9. Male offspring were tested for anxiety-like behavior from PND 53 to PND 55 in the open field test (OF), elevated plus-maze (EPM), and light/dark test (LD). After behavioral testing, animals were euthanized, and glucocorticoid receptor (*Nr3c1)*, corticotrophin-releasing hormone (*Crh*), and its receptor type 1 (*Crhr1*) gene expression in the hypothalamus were measured. Moreover, plasma corticosterone levels were analyzed. We observed that ELS dams presented altered quality of maternal care, characterized by a decrease in arched-back nursing, and an increase in passive nursing. Stressed dams also showed an increase in the number of exits from the nest when compared to CT dams. Furthermore, ELS animals showed increased anxiety-like behavior in the OF, EPM, and LD. Regarding gene expression, we identified an increase in hypothalamus *Crh* levels of ELS group when compared to CT animals, while no differences in *Nr3c1* and *Crhr1* expression were observed. Finally, stressed animals showed decreased levels of

plasma corticosterone when compared to the CT group. In conclusion, we observed an alteration in maternal behavior in ELS dams. Later in life, animals exposed to the combined model of ELS showed increased levels of anxiety-like behavior. Moreover, the central and peripheral HPA measures observed could indicate a dysregulation in HPA function provoked by ELS exposure.

Keywords: maternal separation, limited bedding, HPA axis, anxiety-like behavior, CRH, corticosterone

INTRODUCTION

Over the years, animal studies have been trying to replicate human early life adverse conditions to model the impact of chronic stress exposure during sensitive periods of development. The use of preclinical research is an essential tool to uncover the neurobiological underpinnings associated with early life stress (ELS) exposure. Despite several decades of animal research, ELS studies continue to face challenges, especially because there are controversial and underpowered findings (Tractenberg et al., 2016; Bonapersona et al., 2019; White and Kaffman, 2019; Wang et al., 2020). For example, the meta-analysis of Wang et al. (2020), reported that mice exposed to maternal separation (MS) showed no alteration in anxiety-like behavior, highlighting that MS studies face some uncertainties regarding publication bias (e.g., methodological and experimental procedures inconsistencies) that might affect replication and translational potential of studies. These inconsistencies in experimental ELS protocols may induce distinct degrees of stress exposure, attenuating or exacerbating the effects on both offspring and dams (Lehmann and Feldon, 2000; Tractenberg et al., 2016).

Classical ELS models were designed to separately explore the characteristics of chronic stress early in life. For example, MS is projected to trigger fear in the offspring and dam, mainly due to exposure to an unfamiliar environment (White and Kaffman, 2019). Nevertheless, a past study has shown that maternal care increases post-MS, which could attenuate the long-term effects of MS on behavioral and biological outcomes (Orso et al., 2019). On the other hand, limited bedding (LB) generates high levels of deprivation from acceptable maternal care and resources, which are both necessary for offspring thermoregulation and adequate development (Rice et al., 2008; McLaughlin et al., 2014). We believe that the combination of these protocols is not simple to add stressors, but they can have complementary roles that could increase the challenging conditions imposed to both the dam and the offspring. For example, the pattern of maternal care post-MS reunion could be fragmented by the necessity of the dam to locate resources during LB conditions, which increases the frequency of exits from the nest (Walker et al., 2017). Furthermore, humans that live in stressful environments are usually exposed to multiple stressors simultaneously, and not one negative stimulus exclusively (Evans and English, 2002; Evans and Kim, 2007). Recently, Peña et al. (2017) reported that mice simultaneously exposed to MS and LB showed enhanced susceptibility for the development of depressive-like behavior later in life. However, how a translational approach using the combination of classical ELS models can affect stress-related parameters, such as anxiety-like behavior and hypothalamic-pituitary-adrenal (HPA) axis functioning is yet to be characterized.

Under physiological conditions, the HPA axis is activated to restore cell homeostasis following an acutely stressful event (Kapoor et al., 2008). Briefly, the synthesis and release of corticotrophin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus will stimulate the release of adrenocorticotrophic hormone from the anterior pituitary gland, which causes the adrenal cortex to elevate glucocorticoid levels (cortisol in humans and corticosterone in rodents; Anacker et al., 2014; Jiang et al., 2019). This alteration in glucocorticoid concentration and activation of its receptors (glucocorticoid and mineralocorticoid) triggers a negative feedback mechanism, which reduces the activity of the HPA axis following the stressful event (Herman and Cullinan, 1997; Raubenheimer et al., 2006). These processes are considered essential for the regulation of stress in a healthy organism, but chronic stress may lead to dysfunctions in this stress response mechanism (Dieleman et al., 2015).

Previous studies have shown that CRH and its type 1 high-affinity receptor (CRHR1) play a major role in the pathophysiology of anxiety-like behavior (Müller et al., 2003; Wang et al., 2012). Alterations in CRH levels following ELS exposure have also been associated with long-term cognitive impairments (Ivy et al., 2010). Also, the quality of maternal care, which is usually altered during periods of ELS, appears to have a direct impact on CRH expression (Korosi and Baram, 2009). It has been previously shown that animals reared by dams with increased levels of active arched-back nursing showed reduced CRH levels in the hypothalamus and increased GR expression in the hippocampus when compared to dams with a low frequency of arched-back nursing (Plotsky et al., 1993; Francis et al., 1999; Meaney, 2001).

Despite a growing body of evidence reporting the impact of ELS on anxiety-like behavior and its relation with CRH expression, there are still challenges to improve the translational impact of ELS animal models. Adverse rearing environments in humans are usually characterized by a combination of multiple stressors simultaneously, such as abuse, neglect, household chaos, and lack of resources. However, classical ELS models that have been proposed are insufficient to cover all these conditions. For this reason, we believe that our study will help the field of ELS to move forward by contributing to the validity of the novel ELS model. To do this, we sought to investigate: (i) the effects of MS and LB combined on maternal behavior; (ii) anxiety-like behavior during young adulthood in three tasks (OF, EPM and LD); and (iii) *Crh*, *Crhr1*, and *Nr3c1* mRNA expression in the hypothalamus, as well as peripheral corticosterone levels.

MATERIALS AND METHODS

Animals

BALB/cJ mice were acquired from the colony of the Center for Experimental Biological Models (CeMBE) at the Pontifical Catholic University of Rio Grande do Sul, Brazil (PUCRS). Animals were kept in the animal facility of PUCRS, under controlled temperature 21 ± 1°C in ventilated Plexiglas cages in a 12 h/12 h light-dark cycle (lights on at 7 AM to 7 PM). Animals had access to food and water *ad libitum* during the whole experiment. Mice were bred in-house to avoid any stress associated with animal transportation. Male BALB/cJ mice were housed with two females BALB/cJ for 72. Following this period males were removed, and females were left in pairs for 15 days. Single-housed females were checked twice every day for the presence of pups, and when a pup was found it was considered postnatal day (PND) 0.14 litters were used in total, seven litters were randomly assigned to the control group (CT), and another sevenpotential litter effects a maximum of to the stressed group (ELS). Litters were weaned at PND 21 and weighed at PND 2, PND 16, and PND 53. After weaning, three males from the same rearing protocol were grouped per cage. To avoid potential litter effects a maximum of two males per litter were used in this study, while the exceeded males and all the females were redirected to ongoing projects in the laboratory. Cage cleaning was performed once per week. Mice were tested from PND 53 to PND 55 and were euthanized 30 min after the last behavior test for brain and blood collection. The experimental design is summarized in **Figure 1** (Created with BioRender.com). This study was approved by the local Committee of Ethics on the Use of Animals (CEUA) under #8546 and conducted following the Guide for the Care and Use of Laboratory Animals from the National Institute of Health (NIH).

Early Life Stress Protocol

Animals from the ELS group were exposed to a combined stress protocol from PND2 to PND15 (Peña et al., 2019). The protocol consisted of continuous LB (Rice et al., 2008), where stressed animals were placed in cages with an aluminum mesh on the top of a small number of wood shavings, and were given only 1 g of cotton for nest building. Wood shavings were used for the pups not to get trapped under the aluminum mesh. Animals from the CT group were placed in standard cages with a regular amount of wood shavings and 4 g of cotton for nesting material. After PND15, ELS animals were returned to CT rearing conditions. On top of that, offspring was simultaneously separated from the dams for 3 h per day (9 AM to 12 PM). The dams were removed from the home cage and placed in a new cage, while the pups were individually separated in small containers with little amounts of wood shavings. During separation, the animals were kept in a separate room to avoid possible ultrasonic dam-pup vocalization. Pups were placed on a heating pad with controlled temperature (32 ± 3°C) to prevent stress-induced by hypothermia (Tractenberg et al., 2016). After the MS period, animals were returned to the home cage. Animals from the CT group were left undisturbed during the stress protocol.

Maternal Behavior Observations

Maternal behavior observations were conducted before MS (8 AM to 9 AM), 30 min after MS (1 PM to 2 PM), and 300 min after MS (5 PM to 6 PM). Maternal behavior of all seven litters per group was recorded in intercalated days from PND1 to PND9 (PND1, 3, 5, 7, and 9). The observations were made every 2 min, which consists of a total of 90 observations per day. The following maternal behaviors were recorded: arched-back nursing, passive nursing, licking/grooming, and nest building. The following non-maternal behaviors were recorded: eating, drinking, and self-grooming. Moreover, the number of exits from the nest was recorded during the entire observation period. Two independent observers separately scored maternal behavior at different time points, which were later combined for data analysis. Total maternal and total non-maternal behavior data were calculated using the sum of all individual maternal and non-maternal behaviors recorded. Behaviors are presented by day and time of maternal behavior recording or as the sum of each behavior throughout the blocks and days.

Anxiety-Like Behavior

Open field (OF), elevated plus maze (EPM), and light-dark (LD) were performed between 9 AM to 12 AM to evaluate the anxiety-like behavior of male offspring. To avoid potential confounding effects related to task reactiveness, we performed the behavioral battery in the same order for all animals. Moreover, ten animals per group were subjected to behavioral testing on PND53, PND54, and PND55 respectively. The three tests were video-recorded and later analyzed using the ANY-maze software (Stoelting, Company, Chicago, IL, USA). In every test, the apparatus was cleaned between animals with ethanol 70%.

Open Field Test

Mice were allowed to freely explore a clear acrylic arena (30 × 30 cm) for 10 min under a light intensity of 150 lux. Animals were placed in one corner of the arena to start the test. Using the above-mentioned software, the arena was divided into 16 equal squares, the center four squares were considered the center zone and the remaining squares were considered the periphery zone (Seibenhener and Wooten, 2015). The total distance traveled (in meters), the time spent in each zone, and the number of entries in each zone were analyzed. Decreased time in the center zone and fewer entries in the center zone are associated with increased anxiety-like behavior. Distance traveled was used as a measure of locomotor activity.

Elevated Plus Maze

For the EPM test, animals were allowed to explore a black acrylic apparatus for 5 min (Komada et al., 2008). The apparatus was 50 cm elevated from the ground and consisted of two open arms (30 × 5 cm each) and two closed arms (30 × 5 cm, with a 15 cm wall, each). The test was performed under a light intensity of 40 lux. Mice were placed in the center of the maze heading one of the open arms. The time spent in each arm, the number of entries in each arm, and the number of head

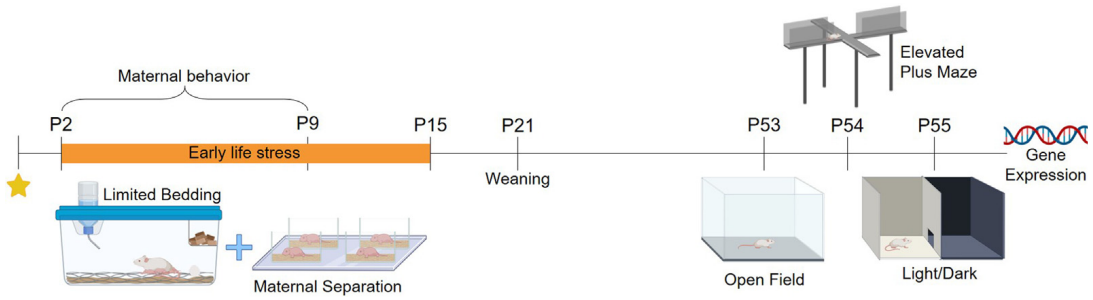

FIGURE 1 | Experimental design and study outcomes.

dips in the open arms was evaluated. Decreased time in the open arms and fewer entries in the open arms are associated with increased anxiety-like behavior. Moreover, a head dip in the open arm to investigate under the maze is considered a risk assessment behavior.

Light/Dark Test

The LD apparatus consisted of two acrylic chambers (21 × 21 × 20 cm), one clear chamber and one black chamber, connected by an open door (Bourin and Hascoët, 2003). The clear chamber was bright (390 lux), while the black chamber was dark. Animals were placed in the bright chamber and had 10 min to freely explore the whole apparatus. The time spent in each chamber, the number of transitions between chambers, and the number of head pokes between the dark and bright chamber were analyzed. Decreased time spent in the light zone and the number of transitions is associated with increased anxiety-like behavior. Furthermore, a head poke occurs when the animal explores a different chamber of the apparatus through the connecting door, which is considered a risk assessment behavior.

Z-Score

The *z*-score combines data from all behavioral tests. The following behavior measures were used: time spent in the center of the OF; time spent in the open arms of the EPM; and time spent in the bright chamber of the LD. In each test the individual *z*-score was calculated using the formula: $(y - x)/z$, where y is the animal score, $\times$ is the average, and z is the standard deviation. Finally, the average of the three *z*-scores was calculated and analyzed. A higher *z*-score represents an increase in anxiety-like behavior.

Gene Expression Analysis

Animals were euthanized by decapitation 30 min after the LD test and the whole brain was removed. The hypothalamus was immediately hand-dissected and snap-freeze in dry ice. The samples were then stored in −80°C for further molecular analysis. Total RNA was extracted from 8 to 10 samples per group using QIAzol (Qiagen; Hilden, Germany) standard protocol. The RNA concentration of each sample was measured with the NanoDrop spectrophotometer. From total RNA, 1.5 μg was reverse transcribed using the miScript II RT Kit (Qiagen). The following Quantitect primers (Qiagen) were used: *Crh* (QT0029389) and *Crhr1* (QT00106232); and the following IDT primers were designed, verified for specificity and used for gene expression: *Nr3c1* (Forward): GGACCACCTCCC AAACTCTG; (Reverse): ATTGTGCTGTCCTTCCACTG; *Pgk* (Forward): TGCACGCTTCAAAAGCGCACG; (Reverse): AA GTCCACCCTCATCACGACCC. SYBR green PCR reactions were run in duplicate for all samples in the Rotor-Gene Real-Time PCR machine (Qiagen). Fold change relative expression was calculated using the ΔΔCt method, using the CT group as a reference and the *Pgk* as an endogenous control for analysis. Due to RNA degradation, two samples were excluded from the CT group. After the analysis of qPCR data, two outliers from the ELS group were excluded from the *Crh* gene expression.

Corticosterone Assay

As a peripheral measure of HPA function, plasma corticosterone levels were measured. Following euthanasia by decapitation 30 min after the LD test, trunk blood was collected and centrifuged to obtain the plasma. A total of 5 μl of plasma was used for analyses in the Corticosterone Enzyme Immunoassay (Arbor Assays) ELISA kit, according to the manufacturer's instructions. The optical density was analyzed at 450 nm wavelength in an ELISA plate reader, and the data was subsequently transformed into concentration (pg/ml) using standard curve parameters. Due to low-quality plasma samples, one animal from each group was excluded during the protocol.

Statistical Analysis

SPSS software v.26 (SPSS, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., CA, USA) were used to perform statistical analysis. Differences between groups (CT × ELS) were analyzed by Student's *t*-test. Shapiro–Wilk test of Normality was performed to verify the normality of data distribution. Pearson's correlation analysis was performed to investigate associations between maternal care, behavioral, and biological data. To investigate the alterations in maternal care throughout the days and different time points, we performed a repeated-measures ANOVA. Tukey's *post hoc* tests were conducted to identify the specific effects in a pairwise comparison. Data are presented as group mean ± standard error of the mean (SEM). Individuals are represented as dots in the graphs, and p-value <0.05 was considered as statistically significant.

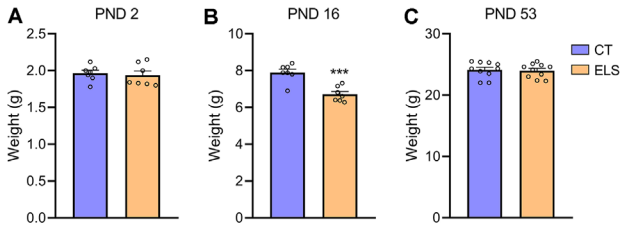

FIGURE 2 | Body weight analysis; **(A)** PND 2; **(B)** PND 16; and **(C)** PND 53. Results are expressed as mean ± standard error of the mean (SEM); $n = 7$ litters per group (PND 2 and PND 16), and 10 animals on PND 53. ***$p < 0.001$ (Student's t-test).

RESULTS

Body Weight

At PND 2 no significant weight difference was observed between groups (**Figure 2A**), but shortly after the end of the stress protocol (PND 16), animals exposed to ELS present a significant reduction in body weight when compared to CT animals ($t_{(12)} = 4.933$, $p < 0.001$; **Figure 2B**). Before the behavioral tasks (PND 53) no weight difference between groups was identified (**Figure 2C**).

Maternal Behavior

Regarding total maternal behavior, stressed dams showed significant increased frequency when compared to CT dams ($t_{(12)} = 3.751$, $p = 0.002$; **Figure 3A**). A repeated measures ANOVA indicated that independent of the day, this increase occurs when maternal behavior was analyzed in the morning (8 AM; $F_{(12)} = 10.055$, $p = 0.008$; **Supplementary Figure 1A**). However, *post hoc* analysis showed that at 1 PM this increase is only present at PND 5 ($p = 0.016$; interaction effect: $F_{(12)} = 5.586$, $p = 0.036$; **Supplementary Figure 1A**), and it is not persistent when maternal behavior was recorded during late afternoon (5 PM). When analyzing nursing behaviors, we observed that arched-back nursing, which is the most effective nursing posture was decreased in stressed dams ($t_{(12)} = 2.800$, $p = 0.016$; **Figure 3B**). A repeated measures ANOVA showed that this decrease in arched-back nursing occurred when maternal behavior was analyzed at 1 PM ($F_{(12)} = 9.519$, $p = 0.009$; **Supplementary Figure 1B**), but for 5 PM, this decrease only was present at PND 7 ($p = 0.003$) and PND 9 ($p = 0.02$; interaction effect: $F_{(12)} = 10.203$, $p = 0.008$; **Supplementary Figure 1B**). Moreover, passive nursing was increased in dams exposed to stress when compared to CT dams ($t_{(12)} = 4.134$, $p = 0.001$; **Figure 3C**). A repeated measures ANOVA indicated that this increase in the ELS group occurred at 8 AM ($F_{(12)} = 5.183$, $p = 0.042$; **Supplementary Figure 1C**), 1 PM ($F_{(12)} = 10.696$, $p = 0.07$; **Supplementary Figure 1C**), and 5 PM ($F_{(12)} = 5.604$, $p = 0.036$; **Supplementary Figure 1C**), independent of the day. Regarding Licking/grooming behavior, we observed a significant increase in stressed dams compared to CT dams ($t_{(12)} = 4.014$, $p = 0.001$; **Figure 3D**). A repeated measures ANOVA showed that independent of the day, this increase occurred both at 8 AM ($F_{(12)} = 13.022$, $p = 0.004$; **Supplementary Figure 1D**), and 1 PM ($F_{(12)} = 12.003$, $p = 0.005$; **Supplementary Figure 1D**), but it was not present at 5 PM Furthermore, we observed that stressed dams showed increased nest building frequency compared to CT dams ($t_{(12)} = 5.887$, $p < 0.001$; **Figure 3E**). A repeated measures ANOVA showed that at 8 AM, this increase was observed independent of the day ($F_{(12)} = 4.88$, $p = 0.047$; **Supplementary Figure 1E**). *Post hoc* analysis revealed an interaction effect for the other time points, which indicated that at 1 PM ($F_{(12)} = 7.011$, $p = 0.021$; **Supplementary Figure 1E**), this increase was present at PND 3 ($p = 0.003$), PND 5 ($p = 0.029$), PND 7 ($p < 0.001$), and PND 9 ($p = 0.010$). While at 5 PM (interaction effect: $F_{(12)} = 7.959$, $p = 0.015$; **Supplementary Figure 1E**), this effect was only present at PND 5 ($p < 0.001$) and PND 9 ($p = 0.004$).

In relation to total non-maternal behavior, stressed dams had a significant decrease compared to CT dams ($t_{(12)} = 2.447$, $p = 0.03$; **Figure 3F**). A repeated measures ANOVA followed by *post hoc* analysis, showed that this increase was only present when non-maternal behaviors were evaluated at 8 AM (interaction effect: $F_{(12)} = 5.710$, $p = 0.034$; **Supplementary Figure 1F**), during PND 5 ($p = 0.01$) and PND 7 ($p = 0.013$), but not effect was observed during any of the afternoon analysis. Moreover, we identified that stressed dams presented a fragmented maternal care pattern by increasing the number of exits from the nest ($t_{(12)} = 11.47$, $p < 0.001$; **Figure 3G**). A repeated measures ANOVA indicated that independent of the day, there was an increase in ELS non-maternal behavior at 1 PM ($F_{(12)} = 45.929$, $p < 0.001$; **Supplementary Figure 1G**). *Post hoc* analysis, revealed that this increase occurred when behaviors were evaluated at 8 AM (interaction effect: $F_{(12)} = 11.584$, $p = 0.005$; **Supplementary Figure 1G**), during PND 5 ($p = 0.001$), PND 7 ($p = 0.005$), and PND 9 ($p = 0.001$). Furthermore, similar effect was observed at 5 PM (interaction effect: $F_{(12)} = 6.844$, $p = 0.023$; **Supplementary Figure 1G**), but only during PND 3 (0.047) and PND 9 ($p < 0.001$).

All correlational analysis between maternal behavior, anxiety-like behavior z-score, and corticosterone levels can be found in **Supplementary Table 1**. We observed that arched-back nursing was negatively correlated with anxiety-like behavior z-score ($r = -0.535$; $p = 0.015$), and positively correlated with corticosterone levels ($r = 0.680$; $p = 0.001$). On the other hand, licking/grooming, nest building, total maternal behavior and exits from the nest displayed positive correlation with anxiety-like behavior z-score ($r = 0.740$, $p < 0.001$; $r = 0.651$, $p = 0.001$; $r = 0.447$, $p = 0.047$; $r = 0.776$, $p < 0.001$, respectively), and negative correlation with corticosterone levels ($r = -0.593$, $p = 0.009$; $r = -0.624$, $p = 0.005$; $r = -0.481$, $p = 0.042$; and $r = -0.717$, $p < 0.001$, respectively).

Anxiety-Like Behavior

Regarding the OF test, there was no significant difference between groups in locomotor activity (**Figure 4A**). However, animals exposed to ELS showed decreased time spent in the center and less entries in the center of the apparatus compared to CT animals ($t_{(18)} = 2.484$, $p = 0.023$; **Figure 4B**; $t_{(18)} = 2.346$, $p = 0.03$; **Figure 4C**, respectively).

In the EPM test, we identified a significant decrease in time spent in the open arms and number of entries in the open arms in ELS animals ($t_{(18)} = 2.409$, $p = 0.026$; **Figure 4D**; $t_{(18)} = 2.512$,

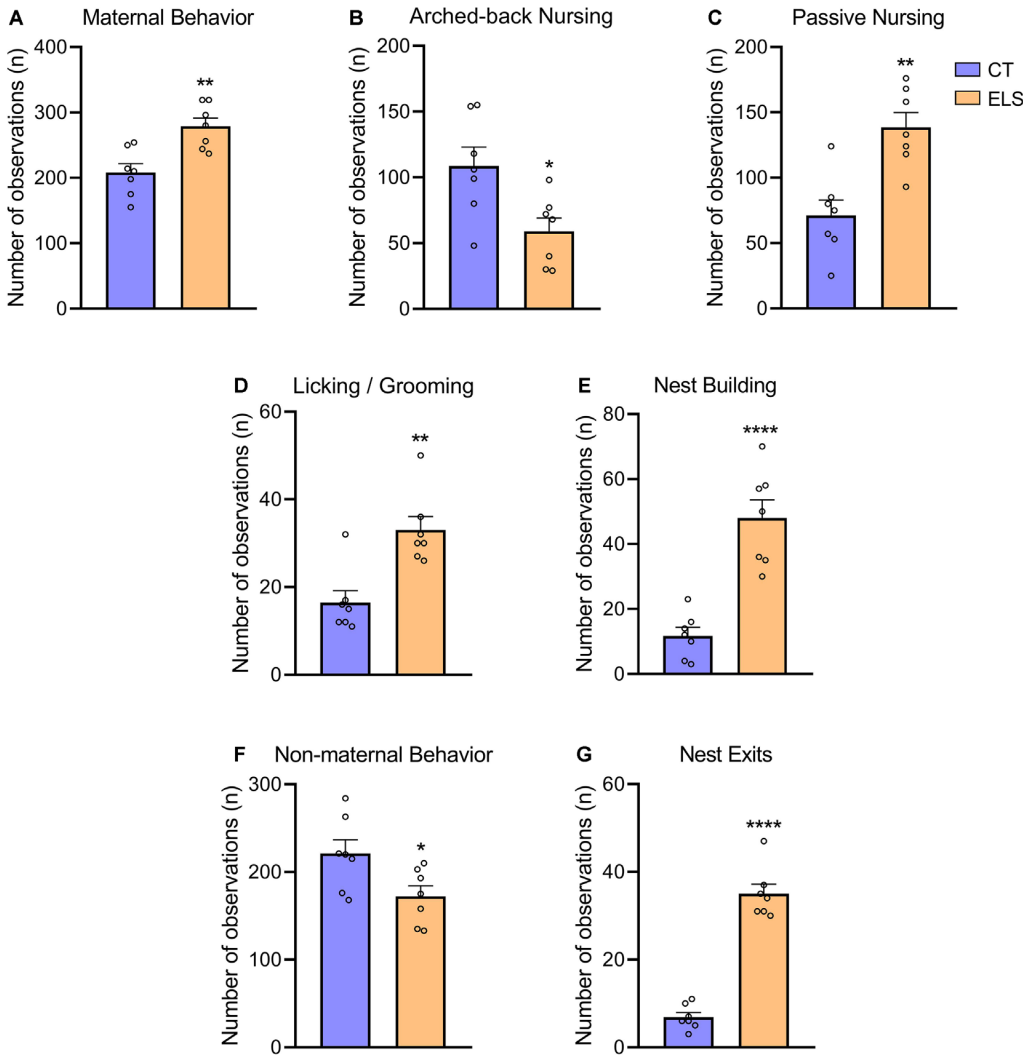

FIGURE 3 | Maternal and non-maternal behavior analysis. **(A)** Total maternal behavior, which takes into account the sum of all maternal behaviors; **(B)** frequency of arched-back nursing throughout the days; **(C)** frequency of passive nursing throughout the days; **(D)** frequency of licking/grooming throughout the days; **(E)** frequency of nest building throughout the days; **(F)** total non-maternal behavior, which takes in account the sum of all non-maternal behaviors; and **(G)** frequency of exits from the nest throughout the days. Results are expressed as mean ± SEM; $n = 7$ litters per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ (Student's t-test).

$p = 0.021$; **Figure 4E**, respectively). The number of head dips in the open arms did not present statistically significant differences between groups (**Figure 4F**).

In relation to the LD test, ELS animals presented a significant decrease in time spent in the light zone, number of transitions and total number of head pokes between zones compared to CT animals ($t_{(18)} = 3.949$, $p < 0.001$; **Figure 4G**; $t_{(18)} = 3.034$, $p = 0.007$; **Figure 4H**; $t_{(18)} = 5.059$, $p < 0.001$; **Figure 4I**, respectively).

Regarding the z-score, which took in account results from all three tests combined, ELS animals showed an increased score compared to CT group ($t_{(18)} = 5.111$, $p < 0.001$; **Figure 5**).

Gene Expression Analysis

When assessing *Crh* mRNA expression in the hypothalamus, we identified an increase in ELS animals compared to CT animals ($t_{(14)} = 2.211$, $p = 0.044$; **Figure 6B**). However, there was no significant difference in *NR3C1* and *Crhr1* mRNA expression between groups (**Figures 6A,C**, respectively).

Corticosterone Analysis

Regarding plasma levels of corticosterone, we identified a significant decrease in animals exposed to ELS protocol compared to the CT group ($t_{(16)} = 4.082$, $p < 0.001$; **Figure 7**). Furthermore, we observed a negative correlation between anxiety-like behavior z-score data and plasmatic corticosterone levels ($r = -0.571$, $p = 0.013$; **Table 1**).

DISCUSSION

This study aimed to investigate the effects of LB and MS models combined on classical anxiety-like behavior tasks and hypothalamic gene expression of targets related to HPA axis

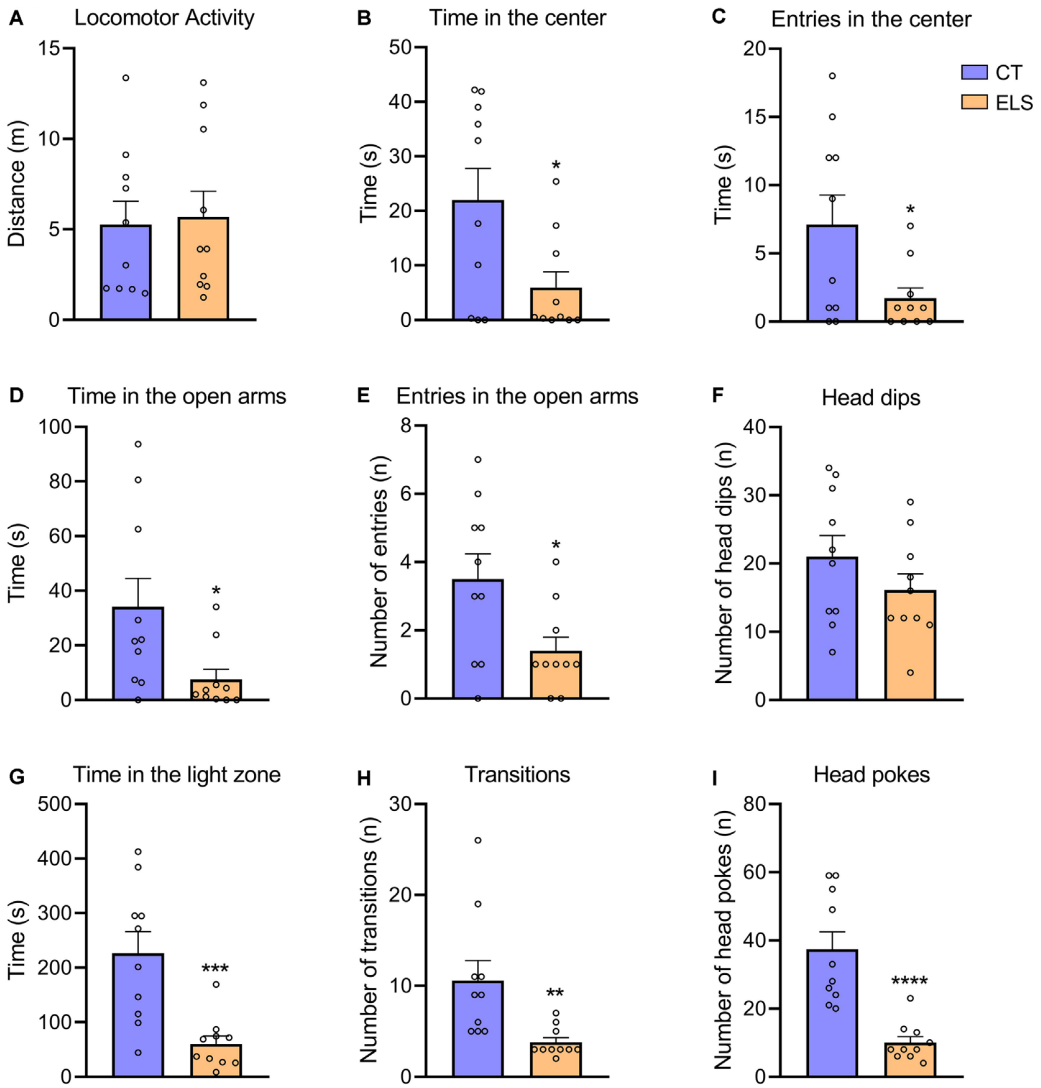

FIGURE 4 | The measure of anxiety-like behavior in the Open Field (OF), Elevated Plus Maze (EPM), and light/dark test (LD). **(A)** Distance exploring the OF apparatus; **(B)** time spent in the center of the OF apparatus; **(C)** number of entries in the center of the OF apparatus; **(D)** time spent in the open arms of the EPM apparatus; **(E)** number of entries in the open arms of the EPM apparatus; **(F)** number of head dips (risk assessment) in the open arms of the EPM apparatus; **(G)** time spent in the light zone of the LD apparatus; **(H)** number of transitions between zones of the LD apparatus; and **(I)** number of head pokes (risk assessment) between zones of the LD apparatus. Results are expressed as mean ± SEM; $n = 10$ per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ (Student's *t*-test).

functioning (*Crh*, *Crhr1*, and *Nr3c1*). Overall, our findings suggested a significant anxious effect of ELS on the main anxiety-like parameters analyzed in the OF (time and entries in the center), EPM (time and entries in the open arms), and LD (time in the light zone, transitions and head pokes). The anxiety phenotype evaluated through the composite *z*-score analysis revealed a higher anxious response of ELS animals in comparison to the CT group. Results from mRNA gene expression revealed significant changes in *Crh* mRNA levels, with increased gene expression in the hypothalamus, while the measure of plasma CORT levels indicated a decrease in ELS animals. Also, our study assessed maternal behavior of dams during early development, in which we observed significant changes in both quantitative and qualitative patterns of maternal care among ELS dams. Especially, we observed an unbalance between arched-back and passive nursing (e.g., decrease of arched-back nursing and increase of passive nursing), despite the increase of licking/grooming, nest building, and total maternal behavior in ELS dams. Moreover, ELS dams presented an increase in the number of exits from the nest, which is considered a fragmented maternal care pattern.

Here, we adopted a new approach to induce a translational and reliable ELS effect by combining MS and LB. The insights for combining ELS protocols derived from discussions expanded by McLaughlin et al. (2014) that proposed a two-dimensional Threat/Deprivation system in quantification and qualification of early stress exposure. Threatening adversities could trigger fear and physical stress, in both relational and environmental

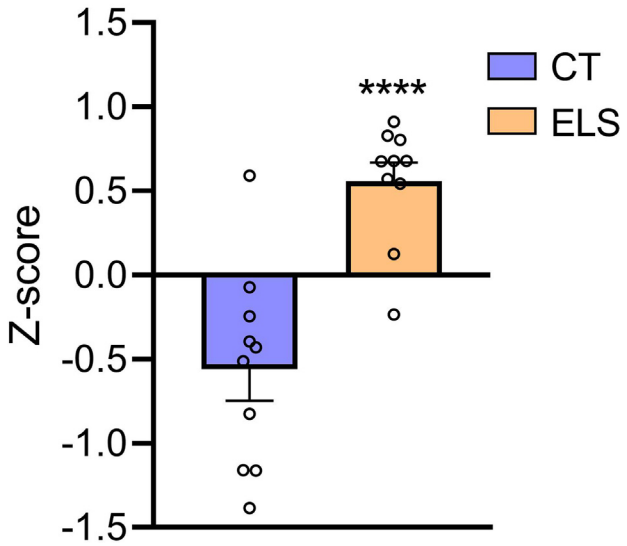

FIGURE 5 | Anxiety-like behavior *z*-score. *Z*-score is calculated using the time spent in the center of the OF, time spent in the open arms of the EPM, and time spent in the bright chamber of the LD. Results are expressed as mean ± SEM; $n = 10$ per group. $^{****}p < 0.0001$ (Student's *t*-test).

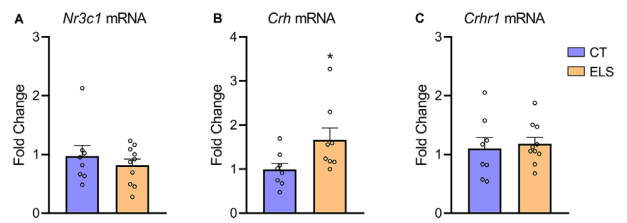

FIGURE 6 | Hypothalamic gene expression of hypothalamic-pituitary-adrenal (HPA) markers. **(A)** *Nr3c1* gene expression; **(B)** *Crh* gene expression; and **(C)** *Crhr1* gene expression. Results are expressed as mean ± SEM; $n = 8$–10 per group. $^{*}p < 0.05$ (Student's *t*-test).

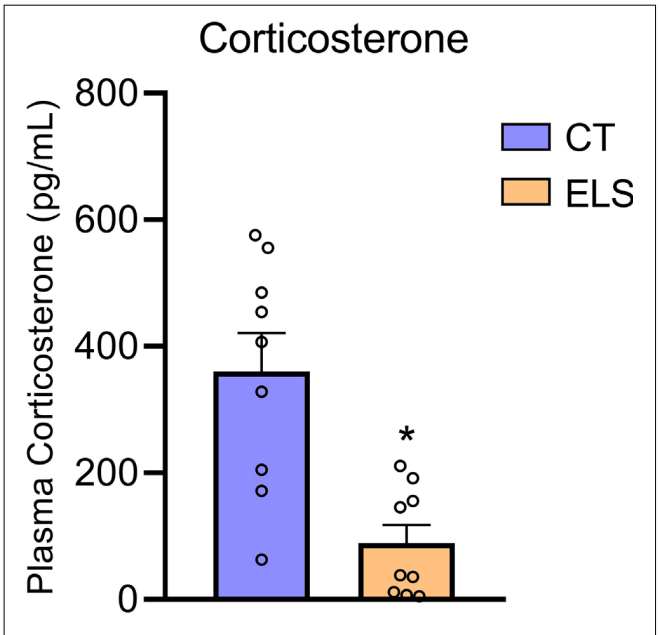

FIGURE 7 | Peripheral HPA response analysis. Plasmatic corticosterone levels. Results are expressed as mean ± SEM; n 6–9 per group. $^{*}p < 0.05$ (Student's *t*-test).

contexts. On the other side, deprivation is related to an insufficient early stimulation, which includes lack of maternal support or appropriate resources and condition for adequate development (i.e., reproducing poverty conditions). In this sense, the combination of both LB and MS could have complementary roles, since LB is suggested to induce significant levels of deprivation and low levels of threat, while MS is associated with intense threat during separation periods (White and Kaffman, 2019). It has already been demonstrated that LB leads to decreased quality of maternal care, which reproduces a lack of early stimulus (Rice et al., 2008). However, during the MS paradigm, the threat tends to raise in response to the novel environment. On the other hand, in a recent review from our laboratory (Orso et al., 2019), we found that MS alone induces an increase in the levels of licking/grooming post-reunion. This counterbalance behavior (increase in maternal care) could attenuate the deprivation characteristics of the MS model, and possibly minimize the effects of the separation period (Davis et al., 2020; Wang et al., 2020). Furthermore, during MS, pups are also exposed to an unknown environment, which is full of novel cues that can also have a direct impact on neurodevelopment. The combination of several factors, such as threat/fear, novel cues, and possible compensatory maternal care displays the challenges in investigating and replicating ELS data in rodents.

Due to the major importance of maternal care for offspring neurodevelopment, we investigated maternal behavior during the ELS protocol. Our analyses suggested significant changes in the pattern of maternal care. Overall, ELS animals showed an increase in the frequency of total maternal behavior, while total non-maternal behavior was decreased. The idea of a compensatory mechanism *via* maternal care alterations can be supported by our data indicating that total maternal care was increased immediately after MS, but it is not persistent during 5 PM analysis. Moreover, an increase in licking/grooming was identified, which corroborates with the compensation hypothesis previously proposed (Orso et al., 2018). These alterations were also followed by an increased number of exits from the nest in animals exposed to ELS, which replicates results usually found in response to LB exposure (Ivy et al., 2008; Walker et al., 2017). Moreover, ELS dams displayed increased nest-building frequency, which indicates an excessive effort to increase nest quality during stressful conditions. We observed that this increase occurred following MS protocol, which reinforces the importance of an adequate nest for pup neurodevelopment. Moreover, ELS dams presented a reduction in the frequency of arched-back nursing, which significantly decreases the quality of maternal care, since this behavior plays a major role in offspring development (Champagne et al., 2003, 2007). Interestingly,

TABLE 1 | Pearson's correlation index between anxiety-like behavior *z*-score, *Nr3c1*, *Crh*, *Crhr*1, and Corticosterone.

	Anxiety-like behavior *z*-score		***Nr3c1***		***Crh***		***Crhr1***		**Corticosterone**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Anxiety-like behavior *z*-score										
Nr3c1	0.025	0.919								
Crh	0.095	0.705	−0.334	0.206						
Crhr1	−0.332	0.177	−0.038	0.880	−0.304	0.251				
Corticosterone	**−0.571***	**0.013***	−0.017	0.945	−0.361	0.168	−0.035	−0.893		

*Note: *p < 0.05.*

this reduced frequency does not only occur immediately after MS (1 PM) but also persists during late afternoon (5 PM) analysis. Our correlational analysis supported this hypothesis, which showed that animals exposed to low levels of arched-back nursing displayed a long-lasting increase in anxiety-like behavior and blunted corticosterone response during early adulthood. Furthermore, we showed that other maternal behaviors, such as nest building and licking/grooming were positively correlated with increased anxiety-like behavior, which could be associated with an attempt of the dam to reduce the deleterious effects of ELS. Thus, using the combination of stressors, we might induce a fragmented and unpredictable maternal care pattern, reproducing a chronic stress condition in both dams and offspring (Walker et al., 2017).

Regarding the behavioral phenotype evaluation, we observed a robust increase in anxiety-like behavior in animals exposed to ELS. The anxiogenic response was identified in all three tasks (OF, EPM, and LD). Moreover, our composite *z*-score analysis sustained the anxiety phenotype as a markable trait in ELS animals. Together these findings corroborate with a recent study, which showed that LB and MS combined resulted in a robust increase in anxiety-like behavior, but LB alone was not sufficient to generate an anxiogenic phenotype (Johnson et al., 2018). Furthermore, several studies have also previously shown that animals reared in LB conditions did not present an increase in anxiety-like behavior during adulthood (Brunson et al., 2005; Rice et al., 2008; Goodwill et al., 2019; Davis et al., 2020; Wang et al., 2012). Those results are not restricted to LB protocol, since they are supported by a range of studies suggesting inconclusive effects of MS. Even though the evidence is indicating that MS exposure may increase anxiety-like behavior (Wei et al., 2010; Malcon et al., 2020), several studies do not replicate those traditional long-term anxiogenic effects following MS (Hulshof et al., 2011; Tan et al., 2017; He et al., 2020). Recently, a systematic review and meta-analytic study reported that there is extensive variability among studies that investigated the impact of MS on OF and EPM tests, which indicates that a better protocol standardization is necessary (Wang et al., 2020).

Animals exposed to ELS showed a blunted corticosterone response 30 min following the LD test. While evidence indicates that ELS exposure can increase corticosterone levels (Rice et al., 2008; Viola et al., 2019), our results may be explained by the high degree of stress applied to the offspring and their dams. This data is supported by a hypothesis which argues that blunted cortisol levels may occur as an adaptation of the sustained periods of HPA axis hyper-reactivity (Trickett et al., 2010; Bunea et al., 2017). Moreover, a recent study with humans reported that ELS severity was negatively correlated with long-term cortisol levels, which shows that a severely stressful event early in development could indeed lead to a blunted cortisol response during adulthood (Zhang et al., 2019). Here, we also showed that plasmatic corticosterone levels were negatively correlated with the anxiety-like behavior *z*-score, which indicates that blunted corticosterone levels were associated with increased anxiety like-behavior. This result could indicate that maintaining adequate corticosterone levels is required to cope with the challenges generated by the behavioral tasks (Cohen et al., 2006). Moreover, previous data have shown that blunted corticosterone levels were associated with HPA axis malfunctioning, which is known to be critical during fight-or-flight situations (Algamal et al., 2018). Furthermore, we also observed an increase in the hypothalamic *Crh* mRNA levels of ELS animals. Following our data, a previous study reported that HPA axis overactivation through ELS exposure may lead to a long-term increase in CRH levels in both the hypothalamus and amygdala (Lai and Huang, 2011). Furthermore, it has been shown that CRH overproduction in transgenic unstressed mice resulted in increased anxiety-like behavior (Stenzel-Poore et al., 1994; van Gaalen et al., 2002). Furthermore, naive CRHR1 knockout animals have been associated with reduced anxiety-like behavior (Smith et al., 1998).

Certain limitations should be considered when interpreting the current work. First, due to the novelty of this ELS model, we opted to perform this study only with male animals. We acknowledge the importance of investigating ELS sex-related differences, so once this combined model of MS and LB is better understood, future studies should seek to investigate neurobiological and behavioral sex differences. Second, even though our study showed a robust effect of the combined ELS model on anxiety-like behavior, it would be important to investigate a direct comparison between MS and LB alone and the combined model of ELS on anxiety-like behavior. For that reason, a future study that will investigate the differences in anxiety-like behavior provoked by these three models is planned. Finally, our study was performed with a single cohort of animals, in which all three behavioral tests and biomolecular analyses were performed. So, we cannot conclude how ELS independently affected gene expression and plasma corticosterone levels without potential responsiveness effect from the behavioral tasks. This strategy was used to reduce the number of animals utilized (Jonasson, 2005), as indicated by the 3Rs (Replacement, Reduction, and Refinement) principle (Flecknell, 2002). The animals were also exposed from less to most stressful tests to have minimal impact on the behavioral results.

In conclusion, exposure to a combined model of MS and LB provoked a long-term increase in anxiety-like behavior in the OF, EPM, and LD tasks. This combined model also resulted in a fragmented and low-quality maternal care, due to an increase in the number of exits from the nest and decreased arched-back nursing frequency. Moreover, a robust disruption in the HPA axis function was identified. Animals exposed to ELS showed a long-term increase in hypothalamic *Crh* mRNA levels and a blunted plasmatic corticosterone response. We believe that the proposed combined model of ELS may generate a translational and reliable approach to mimic the characteristics of human early life adverse conditions, and increase the replicability of ELS preclinical studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is available in the **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee of Ethics on the Use of Animals (CEUA) of PUCRS.

AUTHOR CONTRIBUTIONS

RO: conceptualization, methodology, investigation, formal analysis, and writing—original draft. KC: conceptualization, investigation, and writing—original draft. EK-F: investigation. LW-S: conceptualization and methodology. ST: writing—original draft and writing—review and editing. RG-O: writing—review and editing, supervision, project administration, and funding acquisition. All authors contributed to the article 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/fnbeh.2020.600766/full#supplementary-material.

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Increases in *Bdnf* DNA Methylation in the Prefrontal Cortex Following Aversive Caregiving Are Reflected in Blood Tissue

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Child maltreatment not only leads to epigenetic changes, but also increases the risk of related behavioral deficits and mental disorders. These issues presumably are most closely associated with epigenetic changes in the brain, but epigenetic changes in peripheral tissues like blood are often examined instead, due to their accessibility. As such, the reliability of using the peripheral epigenome as a proxy for that of the brain is imperative. Previously, our lab has found aberrant methylation at the *Brain-derived neurotrophic factor* (*Bdnf*) gene in the prefrontal cortex of rats following aversive caregiving. The current study examined whether aversive caregiving alters *Bdnf* DNA methylation in the blood compared to the prefrontal cortex. It was revealed that DNA methylation associated with adversity increased in both tissues, but this methylation was not correlated between tissues. These findings indicate that group trends in *Bdnf* methylation between blood and the brain are comparable, but variation exists among individual subjects.

Keywords: *Bdnf*, blood, cross-tissue correlation, DNA methylation, early-life stress, epigenetics, maltreatment, prefrontal cortex

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INTRODUCTION

An aversive environment in early life can lead to numerous complications, including structural alterations to the brain and the stress response system and social and cognitive deficits (Cicchetti and Toth, 2005; De Bellis, 2005; Gould et al., 2012). Additionally, child maltreatment puts one at greater risk for numerous mental disorders, including depression, bipolar disorder, anxiety, and schizophrenia (Gibb et al., 2003; Widom et al., 2007; Li et al., 2016; Bahari-Javan et al., 2017; Kefeli et al., 2018). Epigenetic alterations such as DNA methylation are also a product of early-life adversity (Weaver et al., 2004; McGowan et al., 2009; Roth et al., 2009; Franklin et al., 2010; Unternaehrer et al., 2015). DNA methylation is an epigenetic mechanism marked by a methyl group bound to a cytosine in the DNA sequence, and it is typically associated with decreased gene expression (Moore et al., 2013). These epigenetic changes may be an origin of associated neurological and behavioral alterations.

The *Brain-derived neurotrophic factor* (*Bdnf*) gene is involved in brain development, neuroplasticity, and synaptic transmission, and changes in its gene expression have been associated with numerous mental disorders (Binder and Scharfman, 2004; Zheng et al., 2012; Zheleznyakova et al., 2016). DNA methylation at the *Bdnf* gene has been seen in both brain tissue (in animal

models) and blood (in humans) following aversive caregiving or maltreatment (Roth et al., 2009, 2014; Blaze et al., 2013; Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015; Doherty et al., 2016). Such increased DNA methylation and corresponding reductions in gene expression could be leading to some of the adverse effects associated with child maltreatment.

Our lab has previously demonstrated that aversive caregiving leads to increased DNA methylation at *Bdnf* exon IX but not IV in the prefrontal cortex (PFC) of infant rodents (Roth et al., 2009; Doherty et al., 2019). The PFC is an important region to investigate as both early-life adversity and mental disorders have been linked to functional deficits in this brain region (De Bellis, 2005; Noble et al., 2005; Gould et al., 2012; Rock et al., 2014; Zhou et al., 2015). It is more difficult to observe epigenetic changes in human brains, and most often this is only possible post-mortem. Instead, many human studies observe epigenetic changes in peripheral tissues like blood. For example, increased *Bdnf* DNA methylation at exons I, IV, and VI has been seen in the blood of adults who experienced child maltreatment (Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015). However, researchers are still investigating how reliably epigenetic changes in the blood mirror that of the brain. Some studies suggest that methylation at very few CpG sites in the genome are strongly correlated between blood and the brain (Hannon et al., 2015; Walton et al., 2016; Braun et al., 2019). Others have found correlations in global and gene-specific methylation between blood and brain as well as similar methylation patterns between the two tissues (Ursini et al., 2011; Horvath et al., 2012; Ewald et al., 2014; Kundakovic et al., 2015).

The current study aimed to investigate how closely *Bdnf* methylation patterns in the blood reflect that of the PFC following aversive caregiving. DNA methylation within both of these tissues was examined in infant pups following a scarcity-adversity rodent model of low nesting resources. Because early-life adversity has previously been shown to alter methylation at these exons (Roth et al., 2009; Kundakovic et al., 2015; Doherty et al., 2019) and because their corresponding transcripts appear highly expressed in blood (Aid et al., 2007; Cattaneo et al., 2016), *Bdnf* exons IV and IX were selected for initial investigation. Additionally, methylation correlations between the two tissues were investigated. This research holds valuable insights into the reliability of drawing conclusions about the brain from the epigenome of peripheral tissue.

MATERIALS AND METHODS

Subjects

Male and female Long-Evans pups were bred in-house at the University of Delaware. First-born litters were not used to ensure that all dams were experienced mothers prior to experimentation. The date of birth was designated postnatal day (PN) 0, and litters were culled to 5–6 pups of each sex on PN1. Animals were given food and water *ad libitum*. They were housed in polypropylene cages with ample bedding in a temperature- and light-controlled room. A 12-h light-dark cycle (07:00–19:00) was employed, with all behavioral manipulations performed during the light cycle. All practices were in accordance with and approved by the University of Delaware Animal Care and Use Committee.

A Rodent Model for Aversive Caregiving

The scarcity-adversity model of low nesting resources employed in this study has been previously described and shown to induce more aversive maternal behaviors compared to control conditions (Roth et al., 2009; Blaze et al., 2013; Doherty et al., 2016, 2019). Pups in each litter were randomly split into thirds and placed in either the normal maternal care, cross-foster care, or maltreatment condition. For 30 min each day from PN1 through 7, cross-foster care and maltreatment pups were placed with another dam while the normal care pups remained with their biological dam, only being removed from the home cage to be marked for identification and weighed each day. These other dams were lactating, fed the same diet, and postpartum age-matched to the experimental pups, so as not to impact caregiving (Purcell et al., 2011; Grieb et al., 2018). Data suggest that pups cannot distinguish between their biological mother and age-matched dams fed the same diet (Leon, 1975). The cross-foster care pups were placed with a dam who was given an hour to habituate to a plexiglass chamber with plenty of nesting materials. The pups in the maltreatment condition were placed with a dam with no time to habituate to a plexiglass chamber and inadequate nesting materials, conditions previously shown to elicit aversive behaviors (Roth et al., 2009; Blaze et al., 2013; Doherty et al., 2016, 2019). Video cameras recorded each 30-min session, and a trained observer scored a random subset ($n = 7$ litters) for presence of nurturing (licking and grooming, hovering, and nursing) and aversive (dropping, dragging, roughly handling, actively avoiding, and stepping) behaviors in 5-min bins.

Tissue Collection

Brain and blood extractions were performed on PN8. A subset of animals underwent brain perfusions via a saline (0.9% NaCl) flush to ensure that no blood in the PFC tissue was confounding results. These animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and whole blood was extracted directly from the left ventricle prior to perfusions. In the remaining animals, that were not perfused, whole blood was collected at the neck following decapitation. All blood tissue was collected in tubes containing 50 µl EDTA to prevent coagulation. PFC tissue was extracted and homogenized. Whole blood and PFC were stored in −20°C and −80°C freezers, respectively.

DNA Methylation

DNA was extracted from blood and the PFC and bisulfite converted (Qiagen Inc.). Methylation-specific PCR (MSP) was performed with methylation-specific primers designed for *Bdnf* exons IV and IX (Roth et al., 2009). The comparative Ct method was employed to establish fold change in DNA methylation compared to normal care controls, using tubulin as a reference gene (Livak and Schmittgen, 2001; Pfaffl, 2001).

Statistical Analysis

A two-way ANOVA (infant condition x behavior type) with Sidak's *post-hoc* test was used to analyze maternal behavior.

A one-way ANOVA with Tukey's *post-hoc* test was used to analyze the ratio of aversive to total maternal behaviors across the three conditions. Two-way ANOVAs were used to analyze DNA methylation results with the factors being either perfusion (perfusion or no perfusion) and infant condition (cross-foster or maltreatment) or sex and infant condition. After collapsing across sex and perfusion, unpaired two-sample *t*-tests or Mann-Whitney tests (if there was no homogeneity of variance) were used to compare DNA methylation between maltreatment and cross-foster conditions. One-sample *t*-tests were used to compare DNA methylation in cross-foster and maltreatment groups to a theoretical normal care mean of one. Linear regressions of fold change in DNA methylation were run between the PFC and blood. If there was more than one subject in any group from the same litter, they were averaged to avoid within-litter effects. Significance was set to $p < 0.05$.

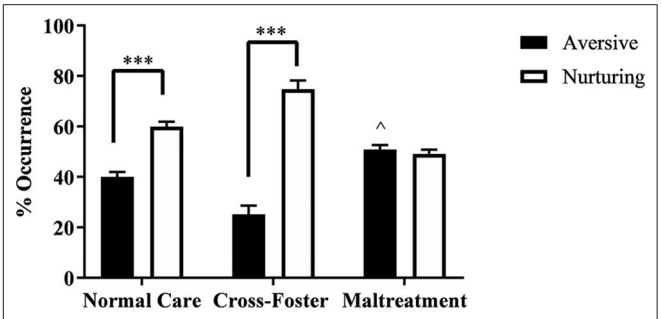

FIGURE 1 | Percent occurrence of aversive and nurturing behaviors across the three infant conditions (normal care, cross-foster care, and maltreatment). ***$p < 0.001$, ^$p < 0.05$ compared to normal and cross-foster care; error bars represent standard error of the mean.

RESULTS

Maternal Behavior

Pups were randomly placed into either the normal maternal care, cross-foster care, or maltreatment condition. A two-way ANOVA examining behavior type (nurturing or aversive) and infant condition (normal care, cross-foster, or maltreatment) revealed a main effect of behavior type [$F_{(1,34)} = 117.6, p < 0.001$] and an interaction [$F_{(2,34)} = 50.41, p < 0.001$]. *Post-hoc* analyses indicated that both normal care ($p < 0.001$) and cross-foster care ($p < 0.001$) conditions had more nurturing behaviors compared to aversive behaviors, while there was no significant difference between nurturing and aversive behaviors in the maltreatment condition (**Figure 1**). A one-way ANOVA examining the aversive behaviors to total maternal behaviors ratio revealed a main effect of infant condition [$F_{(2,17)} = 25.21, p < 0.001$]. *Post-hoc* analyses revealed a significantly higher ratio in the maltreatment condition compared to normal care ($p = 0.022$) and cross-foster care ($p < 0.001$) conditions. Additionally, the cross-foster care condition had a significantly lower ratio compared to normal care controls ($p = 0.002$), an unexpected result; cross-foster and normal care groups typically do not display differences in behavior (Blaze et al., 2013; Roth et al., 2014).

Perfusions

Fold change in DNA methylation compared to normal care controls was calculated for both maltreatment and cross-foster care animals. In two-way ANOVAs (perfusion x infant condition), no main effect of perfusion was found in blood or the PFC at exon IV or IX in males or females (**Supplementary Table 1**). As a result, perfusion and no perfusion groups were collapsed for the remaining analyses.

DNA Methylation Increases in Maltreated Animals

Changes in DNA methylation following aversive caregiving were investigated in both blood and the PFC at exon IV. In the PFC, a two-way ANOVA revealed no main effect of sex or an interaction but a trending effect of infant condition [$F_{(1,124)} = 3.750, p = 0.055$]. One-sample *t*-tests revealed a significant increase in maltreatment males [$t_{(31)} = 2.204, p = 0.035$] and females [$t_{(31)} = 3.287, p = 0.003$] compared to normal care controls (**Figure 2**). When collapsing by sex, the maltreatment group was again significantly above normal care controls [$t_{(63)} = 3.829, p < 0.001$] and trending above cross-foster care animals [$t_{(126)} = 1.924, p = 0.057$].

The DNA methylation in whole blood tissue was similar to that of the PFC at exon IV. A two-way ANOVA revealed no main effect of sex, infant condition, or an interaction. However, one-sample *t*-tests showed that maltreatment males [$t_{(31)} = 2.111, p = 0.043$] and females [$t_{(31)} = 2.532, p = 0.017$] exhibited significantly more methylation than normal care controls (**Figure 3**). Once sexes were collapsed, the maltreatment group was again significantly increased compared to normal care controls [$t_{(63)} = 3.207, p = 0.002$] but not cross-foster care animals.

Methylation changes due to aversive caregiving were also examined at exon IX. In the PFC, a two-way ANOVA revealed no main effect of sex, infant condition, or an interaction, but maltreatment males [$t_{(31)} = 2.635, p = 0.013$] and females [$t_{(31)} = 2.188, p = 0.036$] both exhibited significantly more methylation than normal care controls (**Figure 4**). With sexes collapsed, the maltreatment group was significantly increased compared to normal care controls [$t_{(63)} = 3.044, p = 0.003$] and trending above the cross-foster care group ($U = 1{,}665, p = 0.068$).

Similar methylation changes were seen in the blood at exon IX (**Figure 5**). A two-way ANOVA again revealed no main effect of sex, infant condition, or an interaction. Maltreatment females were trending above normal care controls [$t_{(31)} = 1.879, p = 0.070$], but maltreatment males were not significantly different. However, after collapsing across sex, the maltreatment group was significantly increased compared to normal care controls [$t_{(63)} = 2.191, p = 0.032$], although not compared to cross-foster animals.

DNA Methylation Increases in Cross-Foster Care Animals' Blood

Fold change in DNA methylation in the cross-foster group was also compared to the normal care condition. No significant difference was found between cross-foster care animals and

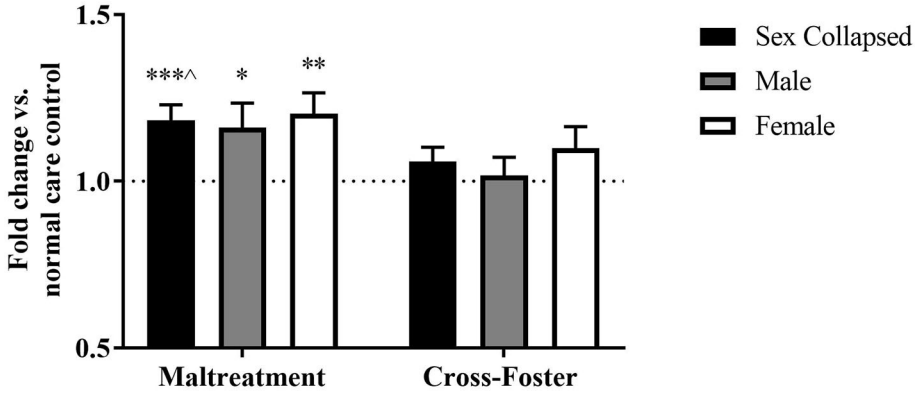

FIGURE 2 | Fold change in *Bdnf* DNA methylation at exon IV in the PFC in the maltreatment and cross-foster groups graphed vs. normal maternal care (dotted line). $n = 12–21$ per group; $^{*}p < 0.05$ vs. normal maternal care (NMC); $^{**}p < 0.01$ vs. NMC; $^{***}p < 0.001$ vs. NMC; $^{\wedge}p < 0.1$ vs. cross-foster care; error bars represent standard error of the mean.

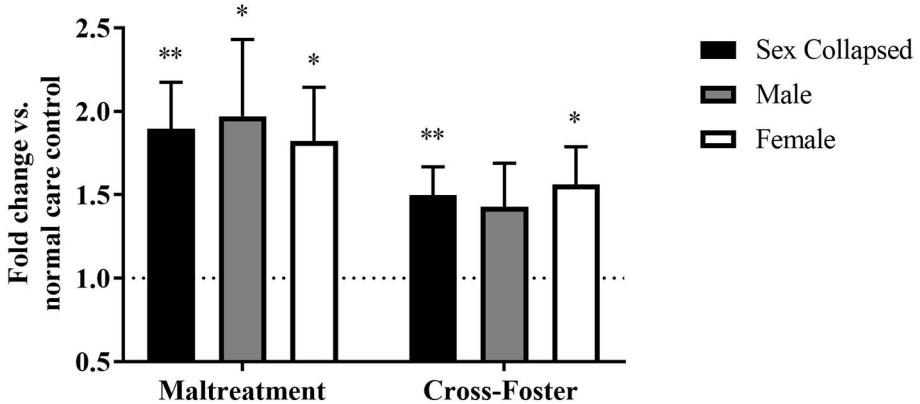

FIGURE 3 | Fold change in *Bdnf* DNA methylation at exon IV in blood in the maltreatment and cross-foster groups graphed vs. normal maternal care (dotted line). $n = 12–21$ per group; $^{*}p < 0.05$ vs. normal maternal care (NMC); $^{**}p < 0.01$ vs. NMC; error bars represent standard error of the mean.

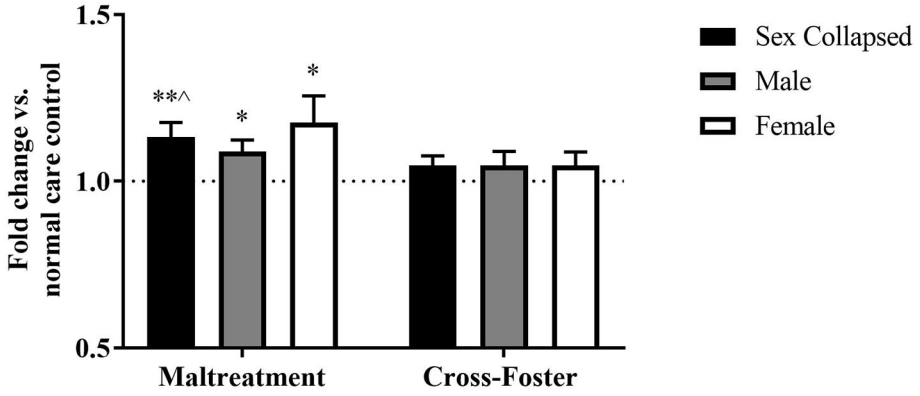

FIGURE 4 | Fold change in *Bdnf* DNA methylation at exon IX in the PFC in the maltreatment and cross-foster groups graphed vs. normal maternal care (dotted line). $n = 12–21$ per group; $^{*}p < 0.05$ vs. normal maternal care (NMC); $^{**}p < 0.01$ vs. NMC; $^{\wedge}p < 0.1$ vs. cross-foster care; error bars represent standard error of the mean.

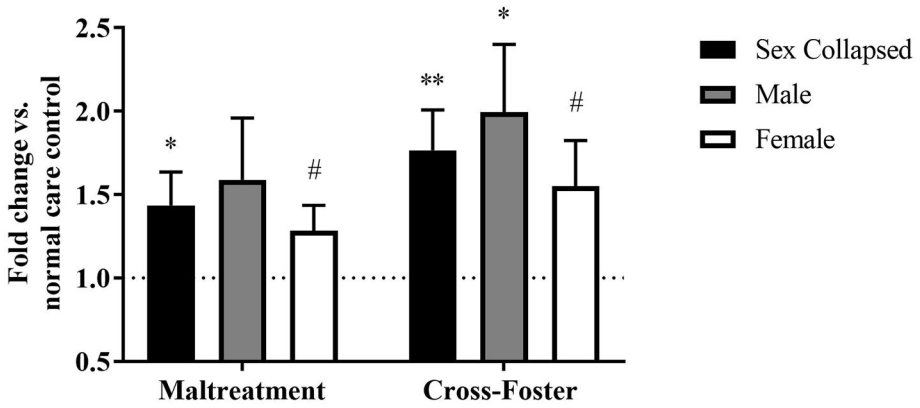

FIGURE 5 | Fold change in *Bdnf* DNA methylation at exon IX in blood in the maltreatment and cross-foster groups graphed vs. normal maternal care (dotted line). $n = 12$–21 per group; $^{*}p < 0.05$ vs. normal maternal care (NMC); $^{**}p < 0.01$ vs. NMC; $^{\#}p < 0.1$ vs. NMC; error bars represent standard error of the mean.

normal care controls in the PFC (**Figures 2**, **4**). However, in the blood at exon IV, significant increases compared to normal care controls were present in cross-foster females [$t_{(32)} = 2.507$, $p = 0.017$] and cross-foster (sex collapsed) [$t_{(63)} = 2.934$, $p = 0.005$] (**Figure 3**). At exon IX in blood, cross-foster females were trending above normal care controls [$t_{(32)} = 2.016$, $p = 0.052$] while males [$t_{(30)} = 2.445$, $p = 0.021$] and the sex collapsed group [$t_{(63)} = 3.166$, $p = 0.002$] displayed significantly increased methylation compared to normal care (**Figure 5**).

Correlations in DNA Methylation Between Blood and the PFC

Linear regressions were run for any group that exhibited the same directional change in methylation in both blood and the PFC (an increase, decrease, or no change in both tissues) (**Figure 6**). At exon IV, this included maltreatment males, maltreatment females, maltreatment (sex collapsed), and cross-foster males. At exon IX, this included maltreatment females and maltreatment (sex collapsed). No significant correlations were found between blood and the PFC for any of these groups. Using a method based on that of Davies et al. (2012), mean difference scores of the log transformed data were analyzed, but also failed to reveal any significant correlations.

DISCUSSION

Since there was no main effect of perfusion in either sex, either exon, or either tissue, it can be concluded that the remaining blood in the rodent brains was not confounding brain methylation results. The blood was presumably in too small a quantity to alter methylation quantification. This suggests future studies exploring methylation in this tissue can gain an accurate reading of brain methylation without performing perfusions.

Once collapsing the data by perfusion, an increase in DNA methylation at exon IV in the maltreatment group compared to normal care controls was found in both tissues. Previously, studies have found no methylation change at exon IV in the infant PFC following this aversive caregiving paradigm (Roth et al., 2009; Doherty et al., 2019). The unexpected increase at exon IV in the current study could be due to the animals being obtained from a vendor not previously used by our lab. Additionally, the sample sizes are larger in this study compared to these previous studies (Roth et al., 2009; Doherty et al., 2019). Although not previously seen in infancy, this paradigm has demonstrated an increase in exon IV methylation in the adult PFC (Roth et al., 2009). It is possible that a minor increase in methylation in infancy becomes a more prominent, and thus more visible, increase in adulthood. Additionally, the human literature has found that exon IV mRNA expression in the PFC does change over the course of early life (Wong et al., 2009), indicating that environmental factors like aversive caregiving may indeed be able to increase exon IV methylation in the PFC at this age.

Aversive caregiving also led to an increase in DNA methylation at exon IV in blood. This is in accordance with previous human studies that have found increased exon IV methylation in the blood of adults maltreated in childhood (Perroud et al., 2013; Thaler et al., 2014). To the best of our knowledge, no studies have investigated such methylation changes in infancy. The current findings suggest that this increased exon IV methylation may occur early in the lifespan.

Aversive caregiving led to increased DNA methylation at exon IV in both tissues, suggesting that methylation alterations within the two tissues may occur in tandem, at least to some extent. This affirms previous research that has found that a rodent model for early-life stress leads to increased exon IV methylation at CpG site 4 in males in the hippocampus and in blood (Kundakovic et al., 2015), along with other studies that have found significant correlations between blood and frontal cortex methylation (Ursini et al., 2011; Horvath et al., 2012). This indicates that blood is an appropriate proxy for the epigenome of the brain in some cases.

After collapsing by perfusion and sex, the maltreatment group exhibited significantly more methylation at exon IX in both the

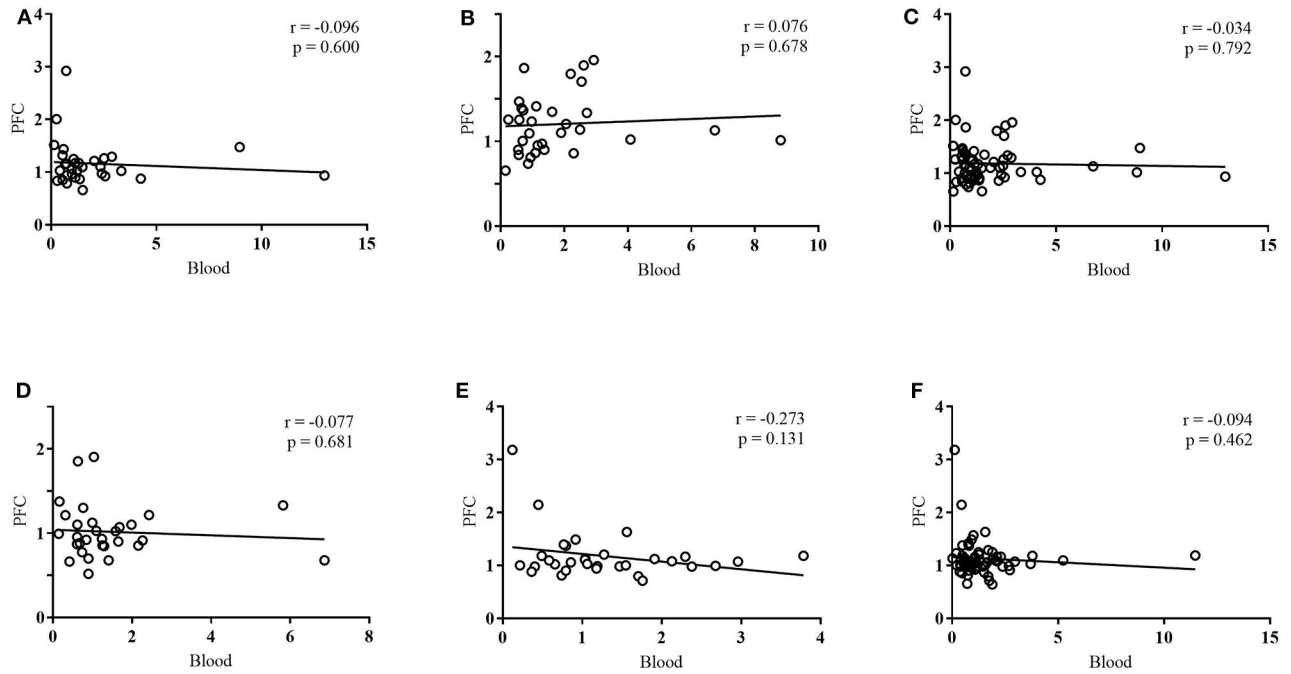

FIGURE 6 | Linear regressions between PFC and blood DNA methylation at **(A)** exon IV in maltreatment males, **(B)** exon IV in maltreatment females, **(C)** exon IV in maltreatment: sex collapsed, **(D)** exon IV in cross-foster males, **(E)** exon IX in maltreatment females, **(F)** exon IX in maltreatment: sex collapsed. DNA methylation was quantified in terms of fold change vs. normal care controls. $n = 12–21$ per group.

PFC and blood. This effect in the PFC was an expected replication of previous findings (Roth et al., 2009; Doherty et al., 2019). To the best of our knowledge, the increase at exon IX in blood brought on by aversive caregiving is a novel finding. However, human studies have previously found child maltreatment to be associated with hypermethylation in blood at exons I, IV, and VI (Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015). Additionally, research has suggested that methylation at exon IX in blood is susceptible to modifications (Hsieh et al., 2019). Just as it did at exon IV, DNA methylation at exon IX appears to follow the same pattern in both the PFC and blood after aversive caregiving. This has important implications for human literature that may be able to rely on using certain methylation changes in the blood as an indication of changes in the brain.

Compared to normal care controls, cross-foster animals exhibited no change in methylation at either exon in the PFC. This is in accordance with previous findings (Roth et al., 2009; Doherty et al., 2019). However, the cross-foster group did show increased DNA methylation at exons IV and IX in the blood. Some shared factor between maltreatment and cross-foster conditions, such as exposure to a novel environment or increased handling, may increase blood *Bdnf* methylation. As such, a cause and effect relationship between aversive caregiving and increased methylation in blood is not explicitly demonstrated. However, as previous literature suggests that blood *Bdnf* methylation increases following early-life adversity (Perroud et al., 2013; Thaler et al., 2014; Kundakovic et al., 2015; Unternaehrer et al., 2015), it is likely that the increase in methylation seen in the maltreatment group is, at least partially, a result of the aversive environment.

Additionally, as gene expression was not explored in this study, it is unknown how the increased DNA methylation in the blood of cross-foster care animals would impact gene expression, if at all. Previously, increased PFC methylation associated with this paradigm has been shown to correspond to decreased gene expression (Roth et al., 2009). However, DNA methylation can also be linked to increased gene expression (Blaze and Roth, 2013). If this is the case in the cross-foster care group, a similar increase in methylation in both conditions could lead to opposite effects on gene expression. Future studies should examine mRNA levels to assess how methylation changes in both cross-foster and maltreatment groups may be impacting gene expression.

No correlations were found in DNA methylation between blood and the PFC at either exon. This suggests that there is no clear-cut correlation in *Bdnf* DNA methylation between the two tissues following aversive caregiving. Despite observing the same group trends in DNA methylation between PFC and blood, there does not appear to be uniformity between tissues at the individual subject level. Additionally, if correlations between the two tissues had been significant, conclusions would have been limited as only select groups exhibited the same directional change in both tissues.

The current study used a method of methylation quantification that allows for methylation to be assessed at individual exons, but not at individual CpG sites within those

exons. Previous reports that have found significant correlations between the two tissues have analyzed methylation at individual CpG sites (Ursini et al., 2011; Horvath et al., 2012; Witzmann et al., 2012; Ewald et al., 2014; Hannon et al., 2015; Kundakovic et al., 2015; Walton et al., 2016; Braun et al., 2019). Quantifying methylation at this level in the future may elucidate site-specific correlations that are otherwise impossible to detect. This is quite likely, as only select CpG sites within the genome appear strongly correlated between blood and the brain (Hannon et al., 2015; Walton et al., 2016; Braun et al., 2019). Additionally, correlations between the two tissues have been found when comparing different CpG sites in each tissue (Ewald et al., 2014), another result that would have been impossible to detect using the method employed in the current study. In the future, examining methylation at individual CpG sites would be valuable in further understanding how these tissues may be correlated.

In addition to exploring methylation at individual CpG sites, future studies may sort cells before assessing methylation changes. As both PFC and blood tissue are made up of multiple cell types, some cell populations may be more closely correlated with the opposing tissue than others. Additionally, blood cell proportions can change due to stress (Dominguez-Gerpe and Rey-Mendez, 2001). Cell sorting would allow us to detect any such proportional changes and the impact this may have on methylation levels, as different blood cells are known to exhibit varying degrees of methylation (Jaffe and Irizarry, 2014). Thus, apparent changes in blood methylation following aversive caregiving may be two-fold: a result of increased methylation in certain blood cells and a result of increased quantities of blood cells that naturally have greater methylation than others.

This study compared PFC and blood methylation at one gene impacted by early-life adversity, but future studies should explore other genes. Studies could also compare blood methylation to other brain regions, as methylation differences have been found between different regions of the brain (Roth et al., 2014). Some research suggests that methylation in saliva may bare a closer resemblance to the brain compared to blood (Smith et al., 2015; Braun et al., 2019). Future studies could explore methylation in saliva to see if this is the case after aversive caregiving. Further research could also explore methylation changes in adult rodents. After aversive caregiving, adult rodent brains have some methylation markers that were present in infancy and some novel methylation markers that arise later in life (Roth et al., 2009, 2014). This may also be the case in the blood. Knowing how blood and brain methylation compare throughout the lifespan would be critical when using blood as a proxy for the epigenome of the brain. The experimental design currently employed does not allow for an analysis of the degree to which individual animals' methylation values correlate to their caregiving experiences. Future studies should also adjust the experimental design so that this analysis can be performed.

Overall, this study found that aversive caregiving alters blood and brain DNA methylation at *Bdnf* exons IV and IX in a similar vein at the group, but not necessarily the individual, level. No correlations were found in methylation between the two tissues. Although further research is needed to more thoroughly understand the relationship between epigenetic changes in these two tissues, there is potential for blood to serve as a proxy for epigenetic changes of the brain following aversive caregiving.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Delaware Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HBDD and TLR designed the study and took part in interpretation of the results. Animal generation, tissue collection, and biochemical and data analyses were performed by HBDD and overseen by TLR. HBDD wrote the first draft of the manuscript. TLR edited the manuscript. Both authors contributed to the article 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/fnhum.2020.594244/full#supplementary-material

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Early Life Stress Predicts Depressive Symptoms in Adolescents During the COVID-19 Pandemic: The Mediating Role of Perceived Stress

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Background: Exposure to early life stress (ELS) is alarmingly prevalent and has been linked to the high rates of depression documented in adolescence. Researchers have theorized that ELS may increase adolescents' vulnerability or reactivity to the effects of subsequent stressors, placing them at higher risk for developing symptoms of depression.

Methods: We tested this formulation in a longitudinal study by assessing levels of stress and depression during the COVID-19 pandemic in a sample of adolescents from the San Francisco Bay Area (N = 109; 43 male; ages 13–20 years) who had been characterized 3–7 years earlier (M = 5.06, SD = 0.86 years) with respect to exposure to ELS and symptoms of depression.

Results: As expected, severity of ELS predicted levels of depressive symptoms during the pandemic [$r(107) = 0.26$, $p = 0.006$], which were higher in females than in males [$t(107) = -3.56$, $p < 0.001$]. Importantly, the association between ELS and depression was mediated by adolescents' reported levels of stress, even after controlling for demographic variables.

Conclusions: These findings underscore the importance of monitoring the mental health of vulnerable children and adolescents during this pandemic and targeting perceived stress in high-risk youth.

Keywords: early life stress, COVID-19, adolescence, depression, perceived stress

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INTRODUCTION

A growing body of research is documenting the adverse consequences of exposure to early life stress (ELS) for mental health across the lifespan (McLaughlin, 2016; LeMoult et al., 2019b). In particular, ELS has been implicated in the elevated rates of depression during adolescence (LeMoult et al., 2019b). Indeed, recent epidemiological data from over 100,000 adolescents ages 12–17 years indicate an incidence of 25% for Major Depressive Disorder (MDD), with females exhibiting about twice the incidence of MDD as males (Breslau et al., 2017); further, almost half

of adults with depression have their first episode during adolescence (Birmaher et al., 2002). Importantly, adolescents who experience depression are significantly more likely as adults to have more mental and physical health illnesses, lower levels of educational attainment, lower salaries, more difficulties in their relationships, and more contact with the criminal justice system (World Health Organization, 2012).

Given the alarmingly high prevalence of ELS (Child Welfare Information Gateway, 2019; Kim and Drake, 2019) and its significant impact on mental health in youth, it is critical that we elucidate mechanisms by which ELS increases risk for depression. One possibility is that exposure to ELS increases individuals' reactivity to subsequent stressors and, thus, also the likelihood that they will experience depression (Post, 2007). Indeed, the odds of reporting psychological distress increase steeply with the number of prior adverse experiences, suggesting that earlier stressful experiences potentiate the impact of later stressful experiences (Manyema et al., 2018). This possibility is especially salient now, given the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or COVID-19) pandemic, which has resulted in personal and familial distress, economic hardship, and enforced social isolation (Galea et al., 2020; Holmes et al., 2020). Indeed, youth are experiencing significant disruptions in their normal daily routines, their face-to-face, peer interactions, and are likely to be particularly susceptible to the stress caused by COVID-related changes and restrictions (Gunnell et al., 2020; Orben et al., 2020). Already, and perhaps not surprisingly, early reports show that 83% of youth (younger than 25 years) with a history of mental illness believe that their conditions are worsening (Lee, 2020). It is unclear, however, whether and how the effects of the pandemic will affect adolescents who do not have significant psychiatric histories, and relatedly, whether exposure to ELS will render adolescents more vulnerable to the stress of the pandemic. Identifying groups of young individuals who experience significant distress in the face of such a stress will facilitate the efficient deployment of prevention efforts such as screening for mental health problems, and in the mobilization of supports such as psycho education and online or remote interventions for those at risk for developing depression and other mental health difficulties (Pfefferbaum and North, 2020). In this paper, we report findings from a longitudinal study in which we examined whether adolescents who experienced more severe ELS as children report higher levels of depressive symptoms during the pandemic, and whether this association is mediated by elevated levels of perceived stress during the pandemic.

MATERIALS AND METHODS

Participants and Procedures

From 2013–2016, we recruited 214 children and adolescents and their parents from the San Francisco Bay Area using media and online advertisements to participate in a longitudinal study of the psychobiological effects of ELS across the transition through puberty (King et al., 2019; LeMoult et al., 2019a; Miller et al., 2020). Inclusion criteria were that adolescents were ages 9–13 years and were proficient in spoken English. Exclusion criteria included factors that would preclude an MRI scan (e.g., metal implants, braces, and claustrophobia), any history of major neurological or medical illness, severe learning disabilities, and, for females, the onset of menses. Following recruitment, four participants were excluded from the final sample (2 withdrew, 1 had a medical illness, and 1 did not respond after initial contact), resulting in 221 participants available for the current study. At baseline, we assessed ELS and depressive symptoms in all adolescents (see section "Measures"). Between April 3 and April 23, 2020, approximately 3 weeks (M = 22.57 days, SD = 4.94 days) after shelter-in-place was implemented in the Bay Area on March 16–18, 2020, adolescents completed online questionnaires assessing depressive symptoms, perceived levels of stress, and exposure and impact of COVID-19 on their behaviors and emotional well-being (see **Figure 1** for a timeline of this assessment). Of the 221 eligible participants, 109 (49.32%) completed the survey [43 males; ages 12.82–19.98 years (M = 16.30, SD = 1.47)]. In accordance with the Declaration of Helsinki, participants and their parents provided informed written assent and consent, respectively. This study was approved by the Stanford University Institutional Review Board and all participants were compensated for their participation.

Measures

Baseline Assessment: Early Life Stress

In addition to obtaining other information and measures at the time of study enrollment, all participants were interviewed at baseline, 3–6.5 years prior to COVID-19 (M = 5.06, SD = 0.86), using a modified version of the Traumatic Events Screening Inventory for Children (Ribbe, 1996). Modifications are described in detail in King et al. (2017); briefly, participants

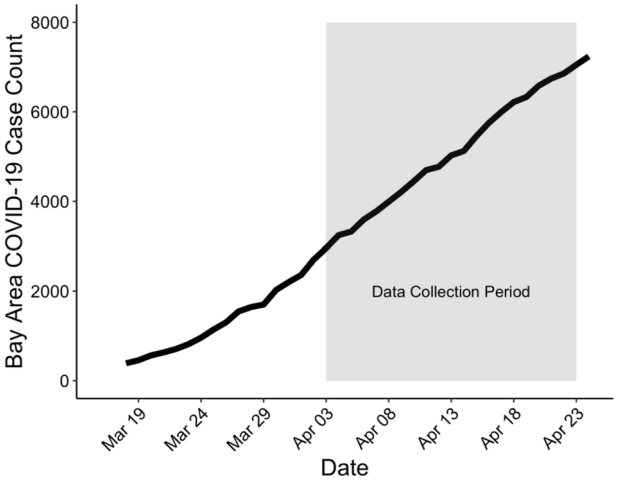

FIGURE 1 | Timeline of data collection in the current study (highlighted in gray) relative to rising cases in the San Francisco Bay Area. Shelter-in-place was issued between March 16–18, 2020. The first participant completed the survey April 3rd, 2020 and the last participant completed the survey April 23rd, 2020. COVID-19 cases were taken from https://data.ca.gov/dataset/covid-19-cases from the following counties: Alameda, Contra Costa, Marin, Napa, San Francisco, San Mateo, Santa Clara, Solano, and Sonoma.

were asked whether they had experienced any of over 30 different types of stressful events (e.g., "Have you ever experienced a severe illness or injury?," "Have your parents ever separated or divorced?"). If a participant endorsed having experienced a stressful event, interviewers followed up with specific questions to contextualize the event (e.g., timing, duration, frequency, and involvement of others). For each event that a participant endorsed, a panel of three coders, blind to the participant's reactions and behaviors during the interview, rated the objective severity of the event using a modified version of the UCLA Life Stress Interview coding system (Rudolph et al., 2000) based on a 5-point scale. To compute the severity of ELS exposure for each participant, we summed the maximum objective severity rating score for each type of stressor endorsed in order to not overweight reports of frequent but less severe events (King et al., 2017).

Baseline Assessment: Depression Symptoms

At baseline, participants completed the 10-item version of the Children's Depression Inventory (CDI-S; Kovacs, 1985), a self-report measure of depressive symptoms designed for youth ages 8–16 years based on a 30-point scale (M = 1.77; SD = 2.05, reflecting very low levels of depressive symptoms at baseline). Responses capture symptoms during the past two weeks (e.g., "I am sad all the time," "I feel alone all the time"). Studies have reported high validity and reliability for this measure (Allgaier et al., 2012). Across the full sample, internal consistency of the CDI-S was high (Cronbach's α = 0.75).

COVID-19 Assessment: Depressive Symptoms, Stress, and Impact of COVID-19

To assess the impact of COVID-19 and the related quarantine ("shelter-in-place") and isolation (social distancing) directives on health outcomes, we sent online questionnaires to all participants. Adolescents also completed the **Center for Epidemiology Studies-Depression for Children scale** (CES-DC; Weissman et al., 1980) a well-validated measure of depressive symptoms used for older children and adolescents (Faulstich et al., 1986), capturing the age range of our sample during COVID-19. The CES-DC is a 20-item measure based on a 4-point scale; responses capture symptoms experienced over the last week (e.g., "I felt down and unhappy," "I felt like crying"). Participants also completed the **Perceived Stress Scale** (Cohen et al., 1983), a 10-item questionnaire based on a 5-point scale. The PSS is a widely used scale for assessing perception of stress in response to life events. Responses capture symptoms and perceptions experienced over the past month (e.g., "how often have you felt nervous and "stressed," "how often have you found that you could not cope with all the things that you had to do"). In the current sample, internal consistency of the CES-DC and PSS were high (Cronbach's α = 0.91 and 0.84, respectively). One male participant did not complete the PSS. Finally, we administered a subset of items from the youth version of the **Coronavirus Health Impact Survey** (CRISIS v. 0.2[1]), recently developed by researchers at the National Institute of Mental Health and the Child Mind Institute. The CRISIS assesses exposure to and symptoms of COVID-19, impact on behaviors, and emotions/worries. With respect to physical symptoms, we asked participants whether in the last two weeks they have experienced fever, cough, shortness of breath, sore throat, fatigue, or loss of taste or smell.

Statistical Analyses

All data were analyzed using R (R Core Team, 2019). We first compared adolescents' levels of ELS and CDI-S scores at baseline to test differences between adolescents who participated in the COVID-19 assessment and those who did not. We then compared male and female adolescents' scores on the baseline and COVID-19 measures. We conducted bivariate Pearson's correlations, corrected for multiple comparisons, to assess the strength of association among the baseline measures, CRISIS items, and the COVID-19-assessed PSS and CES-DC (**Figure 2**). Finally, to determine whether perceived stress accounted for the association between severity of ELS and CES-DC scores, we conducted a mediation analysis and computed 95% confidence intervals (CI) of the indirect effect of perceived stress with bootstrapping (5,000 resamplings) using the "mediate" function from the *psych* library in R (Revelle, 2017). The mediation analysis controlled for age at baseline, CDI-S scores at baseline, sex, race, parental income, and age at the COVID-19 assessment; we conducted a follow-up moderated mediation analysis to determine whether the pattern of results was the same for male and female participants. All variables were z-scored and all parametric statistical tests were two-tailed (α = 0.05).

RESULTS

Demographic Characteristics and Sex Differences

Demographic characteristics of the sample by sex and statistical tests and significance levels are presented in **Table 1**. Male participants were older than female participants at baseline and at the COVID-19 assessment. Male and female participants did not differ in the severity of ELS at baseline; however, female participants had higher CDI-S scores at baseline. Adolescents were racially and ethnically diverse; 58% self-reported as non-white. Household yearly income ranged from <$10,000 to ≥$150,000. Female participants scored higher on the PSS and CES-DC at the COVID-19 assessment than did male participants.

No participant reported having been exposed to someone diagnosed with COVID-19. There were significant sex differences in self-reported impact of COVID-19 based on responses to the CRISIS. Compared to male participants, female participants reported having more COVID-19-related physical symptoms (e.g., cough, shortness of breath, and loss of taste or smell) [$t(107) = 2.45$, $p = 0.016$] and emotional symptoms (worried [$t(107) = 2.26$, $p = 0.026$]; sad [$t(107) = 3.45$, $p < 0.001$]; nervous and anxious [$t(107) = -2.08$, $p = 0.040$]; fatigued [$t(107) = 3.41$, $p = 0.001$]; lonely [$t(107) = -2.83$, $p = 0.006$]; unable to concentrate [$t(107) = -2.58$, $p = 0.011$]; and more negative thoughts [$t(107) = 2.08$, $p = 0.040$]. Finally, consistent with the

[1]https://github.com/nimh-mbdu/CRISIS

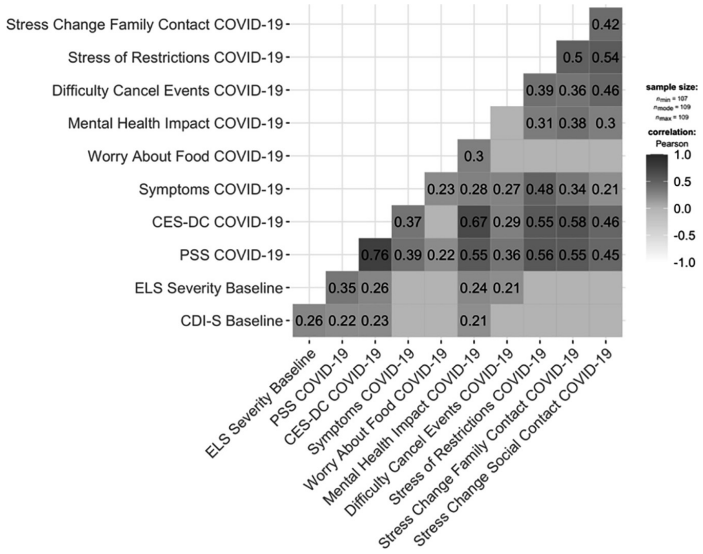

FIGURE 2 | Correlations among Baseline and COVID-19 items/measures assessed in this study. CDI-S, Children's Depression Inventory; ELS Severity, early life stress; PSS, Perceived Stress Scale; CES-DC, Center for Epidemiologic Studies Depression Scale for Children. Content of COVID-19 items: **Symptoms:** self-reported fever, cough, shortness of breath, sore throat, fatigue, loss of taste or smell; **Worry About Food:** "did you worry whether your food would run out because of a lack of money?"; **Mental Health Impact:** "During the past two weeks, how worried have you been about your mental/emotional health being influenced by Coronavirus/COVID-19?"; **Difficulty Cancel Events:** "how much has cancellation of important events (such as graduation, prom, vacation, etc.) in your life been difficult for you in the past two weeks?"; **Stress of Restrictions:** "how stressful have the restrictions on leaving home been for you?"; **Stress Change Social Contact:** "how stressful have these changes in your social contacts been for you?"; **Stress Change Family Contact:** "how stressful have these changes in family contacts been for you?". Correlations are corrected using False Discovery Rate; only significant correlations are shown.

TABLE 1 | Descriptive statistics of study sample by sex.

***Total N* = 109**	**Male (*n* = 43) *M*(*SD*) or *N*(%)**	**Female (*n* = 66) *M*(*SD*) or *N*(%)**	**Statistic**	***p*-value**
Baseline assessment				
Child measures				
Age	11.67 (0.91)	10.95 (0.92)	$t(107) = 3.95$	$p < 0.001$
ELS severity	5.69 (4.50)	5.94 (4.49)	$t(107) = -0.29$	$p = 0.774$
CDI-S total	1.26 (1.22)	2.11 (2.40)	$t(106) = -2.15$	$p = 0.034$
Child race			$X^2(5) = 3.54$	$p = 0.618$
White	20 (47%)	26 (39%)		
Asian/Asian American	7 (16%)	13 (20%)		
Hispanic/Latin-X	3 (7%)	7 (11%)		
Black/African American	3 (7%)	1 (2%)		
Biracial	7 (16%)	15 (23%)		
Other	3 (7%)	4 (6%)		
Parental income			$X^2(3) = 0.60$	$p = 0.897$
<$5,000–$35,000	1 (2%)	3 (5%)		
$35,001–$150,000	19 (44%)	31 (47%)		
$150,000+	20 (47%)	27 (41%)		
Don't know/missing information	3 (7%)	5 (8%)		
COVID-19 assessment				
Age	16.83 (1.22)	15.95 (1.53)	$t(107) = 3.16$	$p = 0.002$
PSS total	15.64 (6.14)	20.24 (6.33)	$t(106) = -3.73$	$p < 0.001$
CES-DC	16.12 (9.24)	23.55 (11.48)	$t(107) = -3.56$	$p < 0.001$

sex differences on the CES-DC, female participants reported that their mental health was more strongly [$t(107) = 2.94$, $p = 0.004$] and adversely affected by COVID-19 [$t(107) = 2.57$, $p = 0.012$], that the cancellation of events was more difficult [$t(107) = 3.60$, $p < 0.001$], and that they worried more that their food would run out due to a lack of money [$t(107) = 2.24$, $p = 0.027$].

Survey Completers Versus Non-completers

Adolescents who completed the COVID-19 assessment did not differ from adolescents who did not complete this assessment in age at baseline [$t(222) = 1.57$, $p = 0.117$]; a lower proportion of Black/African American participants and a higher proportion of Asian/Asian American participants completed the COVID-19 assessment than the baseline assessment [$X^2(5) = 17.72$, $p = 0.003$], but there were no race differences within the group of participants who completed the COVID-19 assessment [$X^2(5) = 3.54$, $p = 0.618$]. Finally, compared to adolescents who did not complete the COVID-19 assessment, adolescents who completed the COVID-19 assessment had less severe exposure to ELS, lower CDI-S scores, and higher parental income at baseline [$t(221) = 2.83$, $p = 0.005$; $t(218) = 2.42$, $p = 0.016$; $X^2(3) = 11.47$, $p = 0.009$, respectively].

Prediction of COVID-19 Perceived Stress and Depressive Symptoms

As hypothesized, severity of ELS at baseline predicted severity of both PSS scores [$r(106) = 0.35$, $p < 0.001$] and CES-DC scores $r(107) = 0.26$, $p = 0.006$] at the COVID-19 assessment (see **Figure 3**). It is also important to note here that rates of stress and depression in this sample at the COVID-19 assessment were high. The mean PSS score in this sample (18.45) is 4.25 points higher than was reported in a validation sample (Cohen et al., 1983), and 63% of our sample scored above the clinical cut-off of 15 on the CES-DC. To examine whether the association between severity of ELS at baseline and depressive symptoms at the COVID-19 assessment was mediated by levels of perceived stress during the pandemic, we conducted a mediation analysis. The mediation model was significant ($R^2 = 0.053$, $p < 0.001$) and indicated that higher PSS scores at the COVID-19 assessment significantly accounted for the positive association between severity of ELS at baseline and CES-DC scores at the COVID-19 assessment (standardized indirect effect: 0.22; 95% CI: 0.07 to 0.36) Importantly, this mediation of the association between ELS and CES-DC scores by PSS was not moderated by sex ($p > 0.05$; see **Figure 4**). Importantly, all of these significant findings held when we controlled for the presence of physical symptoms. Finally, as can be seen in **Figure 2**, the specific COVID-19 stressors that were associated most strongly with perceived stress were those related to changes in restrictions to home confinement [$r(106) = 0.56$, $p < 0.001$], in contact with family [$r(106) = 0.55$, $p < 0.001$], and in social contact [$r(106) = 0.45$, $p < 0.001$]; COVID-related physical symptoms were also associated significantly, but more weakly, with perceived stress [$r(106) = 0.39$, $p < 0.001$].

DISCUSSION

Early life stress (ELS) is a well-documented precipitant of depression later in life (LeMoult et al., 2019b). In particular, stress experienced during early development such as childhood (Teicher et al., 2016) may cause sustained neurobiological changes that increase sensitivity to future stressors (Post, 1992). In this context, the ongoing COVID-19 pandemic is likely to cause widespread difficulties in mental health (Holmes et al., 2020), including depression, particularly among adolescents who are at elevated risk for depression due to having experienced ELS. In the present study, we tested this formulation by leveraging a richly characterized sample of adolescents whom we had been following for several years. We obtained data on levels of depressive symptoms, stress, and contextual factors related to mental health functioning during the pandemic in Northern California, an early epicenter of the pandemic and one of the first areas in the United States to implement isolation directives. Importantly, these adolescents had very low levels of depressive symptoms at the time of recruitment and had been carefully phenotyped with respect to previous exposure to ELS. We found here that greater exposure to ELS predicted higher levels of symptoms of depression during the pandemic. Further, higher levels of perceived stress during the pandemic significantly mediated this association, even after controlling for age, sex, race, income, and earlier levels of depression. Our results highlight the importance of considering early adverse experiences when evaluating mental health risk for adolescents and suggest that psychosocial interventions that reduce the perception of stress and increase social connectedness will mitigate this risk.

Our findings are consistent with early reports from the Hubei province in China, which documented increased levels of depression and anxiety in adolescents during the COVID-19 pandemic (Xie et al., 2020). In addition, emerging research in the United States points to a worsening of mental health in youth with pre-existing mental illness following school closures during the pandemic (Lee, 2020). A key strength of our study, however, is that we *prospectively* predicted mental health functioning during COVID-19 using data we had acquired 3–7 years prior to the pandemic in a sample of adolescents who, at that time, had minimal symptoms of depression. Notably, the adolescents in our full sample who participated in the current COVID-19 study had *lower* levels of depression at baseline than did the adolescents who did not participate and, yet, over 60% of the sample endorsed current levels of depression that warrant additional screening. Thus, our results are likely to be underestimating the adverse impact of ELS on depression risk in adolescents. Moreover, in addition to identifying ELS and female gender as significant risk factors for the development of depression in adolescents during the COVID-19 pandemic, we drew on Post's (2007) formulation of kindling to identify and test a mechanism—perceived stress—that mediates this association and, importantly, that clinicians can target. Finally, we also found that the daily life changes in response to quarantine and isolation directives adolescents reported as being most stressful primarily concerned social stressors. Thus, our study contributes important new findings that can inform clinical practice in identifying and treating at-risk youth and guide public health policies seeking to reduce viral transmission while considering the mental health implications of such directives.

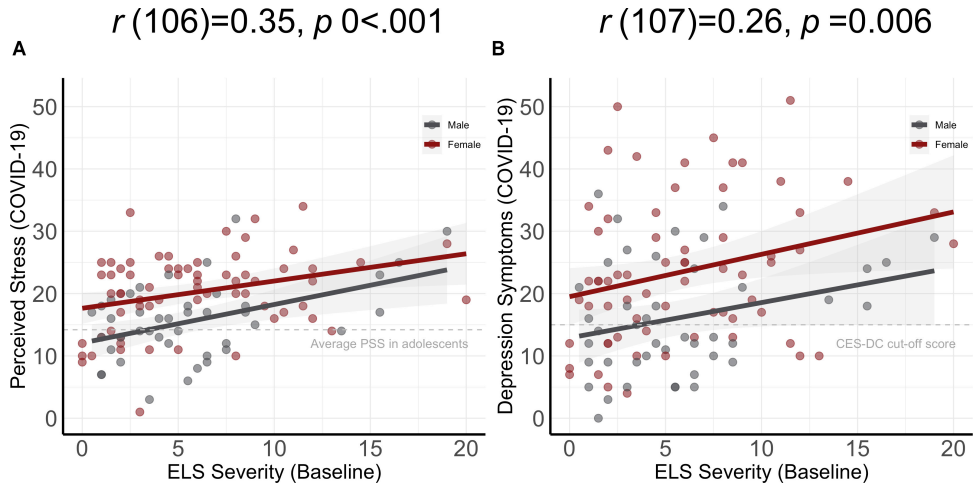

FIGURE 3 | Severity of ELS assessed at baseline is significantly associated with PSS **(A)** and CES-DC **(B)** scores at the COVID-19 assessment. The shaded areas represent 95% confidence intervals of the regression lines.

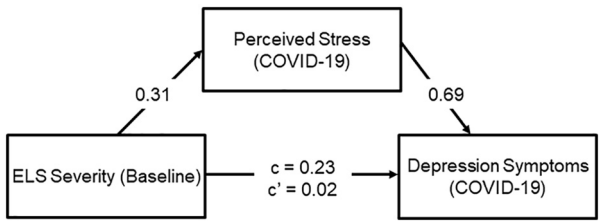

FIGURE 4 | Perceived stress significantly mediates the association between ELS and depression at the COVID-19 assessment. Covariates in this analysis were age at baseline, CDI-S scores at baseline, sex, race, parental income, and age at the COVID-19 assessment. All variables were *z*-scored.

Previous studies have linked higher rates of depression to large-scale crises that affect public and economic health – including earthquakes (Tang et al., 2015), wildfires (Zeller et al., 2015), and terrorist attacks (Liu et al., 2020). The situation generated by the COVID-19 pandemic, however, is unprecedented in terms of its consequences of mandated social isolation (Brooks et al., 2020; Golberstein et al., 2020). The mental health consequences of stressors resulting from extreme social upheaval are almost certain to be compounded by living situation instability, food insecurity, and other financial hardships that many are experiencing during the pandemic. Nevertheless, unlike economic instability, perceived stress is a modifiable target for accessible interventions that may prevent the exacerbation of depressive symptoms during a time when access to standard care and social support is dramatically reduced. For example, cognitive reframing can be implemented *via* telemedicine. Tele-mental health has been shown to be as effective as in-person therapy, including for adolescents and individuals with mood disorders (Slone et al., 2012; Arnberg et al., 2014); such therapies may help adolescents and parents cope with stressors and find meaning in response to a collective trauma, fostering resilience and social connectedness.

We should note limitations of our study. We assessed adolescents during the initial weeks of the Bay Area quarantine and social distancing policies enacted in response to COVID-19; given shelter-in-place directives, however, our data are necessarily based on responses to online self-report measures spanning the time period when quarantine began. In this context, our measures of stress and depressive symptoms during COVID-19 were assessed concurrently. It will be important that future studies examine longer-term effects on adolescents of pandemic-related social restrictions (Andrews et al., 2020). In addition, because depression is one of the most prevalent mental health difficulties in adolescence, we focused in this study on depressive symptoms as a clinical outcome; additional studies are needed to test whether our findings generalize to other mental health conditions (e.g., bipolar disorder, psychosis, and suicidality). Further, only about half of the original participants completed the COVID-19 assessment; importantly, however, completers had less severe exposure to ELS, lower baseline depressive symptom scores, and higher parental income at baseline, making therelatively high levels of stress and depressive symptoms at the COVID-19 assessment all the more striking. The San Francisco Bay Area was one of the first areas to implement widespread and stringent quarantine and social distancing policies; it will be important that researchers assess adolescents in other geographic areas in which similar policies were implemented later and examine whether the duration of social isolation is associated with adverse mental health outcomes. Finally, if we are to foster mental health in a time of a globally stressful event, future studies must also identify sources of resilience that can buffer the onset of depressive symptoms, particularly in high-risk youth.

This study is the first to assess longitudinally the relation between ELS and depression during the COVID-19 pandemic. Our findings indicate that it is critical that adolescents, especially females, who have experienced ELS be monitored regularly for safety and mental health. Certainly, it is too early to determine the long-term impact of the COVID-19 pandemic on mental health. Nevertheless, our study, as well as those from other investigators around the world, shows that high levels of depressive symptoms are likely to occur during the pandemic. We also know that many families will endure financial hardship and an absence of social support even after the quarantine and social isolation policies have been lifted. Thus, our results highlight the importance of implementing accessible psychosocial interventions designed to reduce stress perception and increase social connectedness during this difficult time.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/lrborchers/els_covid.git and https://rpubs.com/lrborchers/707001.

ETHICS STATEMENT

This study was approved by the Institutional Review Board at Stanford University. Participants provided written informed assent and their parent(s)/legal guardian(s) provided written informed consent.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and conduct of the study and to the writing of the manuscript.

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Maternal Distress and Offspring Neurodevelopment: Challenges and Opportunities for Pre-clinical Research Models

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Pre-natal exposure to acute maternal trauma or chronic maternal distress can confer increased risk for psychiatric disorders in later life. Acute maternal trauma is the result of unforeseen environmental or personal catastrophes, while chronic maternal distress is associated with anxiety or depression. Animal studies investigating the effects of pre-natal stress have largely used brief stress exposures during pregnancy to identify critical periods of fetal vulnerability, a paradigm which holds face validity to acute maternal trauma in humans. While understanding these effects is undoubtably important, the literature suggests maternal stress in humans is typically chronic and persistent from pre-conception through gestation. In this review, we provide evidence to this effect and suggest a realignment of current animal models to recapitulate this chronicity. We also consider candidate mediators, moderators and mechanisms of maternal distress, and suggest a wider breadth of research is needed, along with the incorporation of advanced -omics technologies, in order to understand the neurodevelopmental etiology of psychiatric risk.

Keywords: maternal distress, pregnancy, neurodevelopment, psychiatric disorders, pre-clinical models

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The importance of the pre-natal period for neurodevelopment has been well-established through three major research streams. The first involves the follow-up of mothers and offspring subjected to an intense period of adversity during pregnancy. These studies include, amongst others, Hurricane Katrina, the North American Ice Storm of 1998 and mortality of a close relative (see below for references). The strength of these opportunistic, observational studies is the relative uncoupling of the exposure to the relevant event from the circumstances and conditions of the individual families and outcome measures, avoiding issues of reverse causality and supporting conclusions of cause – effect relations (e.g., it is unlikely that another's mental health caused Katrina!). The second stream includes longitudinal, epidemiological studies of mothers and offspring focusing on maternal symptoms of depression and anxiety, as well as measures of maternal stress. The strength of such studies is the prospective design and depth of measurement that permits detailed statistical modeling. These studies include mediational analyses that implicate

candidate biological pathways. The third stream involves studies investigating the effects of stress imposed during pregnancy on developmental outcomes in the offspring of non-human species. The first two streams are descriptive studies of the associations between maternal conditions during pregnancy and neurodevelopmental outcomes in the offspring. The latter stream includes experimental studies that provide "biological plausibility" to support a causal relation between pre-natal maternal conditions and offspring development, as well as to identify candidate mechanisms. Collectively these studies form the basis for our understanding of the importance of pre-natal maternal health and well-being in offspring development.

ETIOLOGY AND CONSEQUENCES OF ADVERSE MATERNAL MENTAL HEALTH IN PREGNANCY

There are two major streams of human epidemiological studies concerning maternal stress which provide very different models of maternal influence on neurodevelopment. The first refers to the sudden exposure to severe conditions such that they can be considered traumatic events. In such instances, pregnant women and their fetuses are exposed to severe adversity, the occurrence of which is independent of the pre-trauma maternal conditions. We appreciate the caveats to this assumption. While the nature and occurrence of the traumatic event may be random with respect to prevailing individual circumstances, the impact of the event is certainly moderated by pre-existing factors such as socio-economic status, social support networks, etc. However, these moderating influences would likewise vary across control populations such that the operative variable is that of the catastrophe. For the sake of this discussion, and with a plea for clemency, we will refer to these studies which examine sudden, catastrophic events as those of "maternal trauma" and those examining more sustained exposure to adverse environmental conditions, as well as symptoms of depression and anxiety, as "maternal distress" (borrowing the term from the original designations by Selye, 1975). We appreciate the clumsiness of this distinction. Trauma breeds lingering states of mental anguish (Yehuda and Meaney, 2018). Elsewhere, we (O'Donnell and Meaney, 2017) and others argue that symptoms of depression, anxiety and perceived stress *should not* be considered as synonymous with respect to either underlying mechanisms or effects on offspring development. Disingenuously, we now ignore this directive. The intent here is simply to note variation in the forms of maternal adversity and to distinguish those with a sudden onset at a specific perinatal period (i.e., trauma) from those that are chronic and, as argued below, generally operative across reproductive life (i.e., distress). We claim no other merit to this terminology. Unfortunately, studies with model systems of perinatal maternal "stress" commonly fail to recognize this important feature to the reality of maternal adversity in humans.

Maternal Trauma

The study of maternal trauma derives largely from epidemiological surveys of the consequences of an environmental (i.e., Hurricane Katrina, Superstorm Sandy and the North American Ice Storm of 1998) or personal (i.e., the loss of a close relative) catastrophe. These are characterized by an acute onset of extreme trauma, which can be used to identify critical periods for psychiatric risk during gestation. For instance, in a large Danish population study of 1.38 million births, death of a close maternal relative, specifically during the first trimester, was associated with an increased incidence of schizophrenia in offspring (Khashan et al., 2008). Similarly, an increased risk of schizophrenia was seen in Dutch infants who were in their first trimester during the 1940 German invasion, with a male specific effect seen in the second trimester (Van Os and Selten, 1998). A pronounced effect on male fetuses was also seen following the 9/11 terror attacks, where across America there was an increased number of females born relative to males suggesting a selective loss of male fetuses (Bruckner et al., 2010). Similar effects were seen in the aftermath of the Chernobyl nuclear meltdown, but with a selective loss of males who were in their third month of gestation at the time of the incident (Peterka et al., 2004). Other environmental catastrophes such as the ice storm of 1998, superstorm Sandy and hurricane Katrina have been associated with reduced cognitive and language skills in offspring, as well as changes in infant temperament compared to pregnancies carried outside these periods (Laplante et al., 2008; Tees et al., 2010; Zhang et al., 2018).

Interestingly, trauma during the pre-conception period also confers an increased risk for adverse pregnancy outcomes. A large Swedish population study demonstrated that the loss of an immediate maternal relative 0–6 months *before conception* was associated with an increase in infant mortality during the first year of life, with no effect seen if bereavement occurred post-conception (Class et al., 2013). Bereavement or severe illness in a close relative during the pre-conception period can also increase the risk of preterm birth (Khashan et al., 2009), which is independently associated with atypical neurodevelopment (Johnson and Marlow, 2017).

It should be noted there are 2 components which dictate the response to trauma; objective hardship and subjective stress. Both of which disproportionately affect those in lower socio-economic brackets (Mcewen and Gianaros, 2010; Ursache et al., 2015). These risk factors overlap with those for chronic maternal distress, but subjective stress is derived from genetic components which are distinct from instances of chronic distress, such as depression (Rietschel et al., 2014).

Maternal Distress

Anxiety and depressive disorders are pervasive throughout society but women, particularly during their reproductive years, are more likely to be affected (Seedat et al., 2009; Parsons et al., 2012). Of particular note is that anti-depressant use during pregnancy has been associated with adverse birth outcomes (Huang et al., 2014) and altered brain connectivity in offspring (Lugo-Candelas et al., 2018). However, it should be noted this is not seen in all studies (El Marroun et al., 2014). Nonetheless, many women go untreated for depression during pregnancy, likely contributing to a more severe psychopathology (Geier et al., 2015; Byatt et al., 2016). Data from the Avon Longitudinal Study

of Parents and Children (ALSPAC) show that maternal anxiety and depression are associated with persistent and clinically meaningful alterations in behavior and emotionality of offspring (O'Donnell et al., 2014), as well as psychotic experiences by 18 years of age (Srinivasan et al., 2020). ALSPAC analyses reveal that the well-established association between maternal depression with that for the offspring (Weissman et al., 2006) is largely attributable to the pre-natal maternal condition (Pearson et al., 2013). The association between maternal depression and cognitive outcomes in the offspring shows the same pre-natal dominant influence (Evans et al., 2012).

Meta-analyses indicate an incidence of 15.2% for any anxiety disorder (Dennis et al., 2017) and 7.4-12.8% for depression (Bennett et al., 2004) during pregnancy. It is also likely these numbers are an underestimation, as a significant portion of mood disorders during pregnancy go undiagnosed (Dietz et al., 2007; Ko et al., 2012). Perinatal mental health disorders are also more common in lower and middle income countries (Fisher et al., 2012), likely a result of the social and economic factors which may influence chronic stress during pregnancy. In contrast, the incidence of common drivers of maternal trauma, death or diagnosis with a serious medical condition in a close relative, ranges from 1.6 to 2.7% (Khashan et al., 2009; Class et al., 2013). Therefore, a better understanding of the fetal effects of chronic distress is, unequivocally, an important public health issue.

Animal Models

Considering the inevitable confounders present in human cohorts (i.e., co-morbidities and socio-economic factors), ascribing a specific event or perturbation to a subsequent outcome can be difficult. To this end, animal models have been fundamental to the establishment of a causal relationship between pre-natal stress and behavioral outcomes. Initial animal models used conditioned aversive stimuli to induce distress in pregnant rodents (Thompson, 1957), with subsequent experiments using a variety of physical and sensory stimuli including physical restraint (Ward, 1972) and electric shocks (Takahashi et al., 1998). Many stimuli can be used to elicit distress in rodents including; restraint, bright light, housing in a small cage, white noise, predator noises or scents, sleep deprivation, forced swimming or exposure to a lactating female (reviewed thoroughly in Weinstock, 2017). Chronic variable stress (CVS) is a common model of pre-natal stress and uses combinations of these stimuli, staged at random points during the day to avoid habituation. These models are typically only used for a period of pregnancy (∼1 week) and therefore are not designed to investigate chronic distress, associated with persisting adverse mental health, but rather the timing and critical periods associated with acute pre-natal stress exposure. This feature of the experimental design is unquestionably important to understand, but as we will discuss later, during pregnancy, maternal distress is more likely to be driven by chronic stressors that remain stable over longer periods of time, to which current CVS models have less obvious parallels. Considering social stress is likely to have a central role in instances of human chronic distress, the clear bias toward physical stressors is also a potential limitation in these studies. Future studies may also consider the inclusion of social stressors in these paradigms (e.g., social defeat or isolation).

Models akin to CVS are used to induce depressive and anxiety-like behaviors in non-pregnant adult rodents (Lezak et al., 2017), and do recapitulate gene expression patterns associated with human depression (Scarpa et al., 2020). However, these depression models are used for several weeks (Wang et al., 2017) and variability in results using this model has been ascribed to shorter paradigm durations (Willner, 2017), indicating a longer period of exposure is needed to reproducibly induce depressive and anxiety-like behaviors in adult animals. Importantly chronic stress exposure in pregnant mice results in anxiety and depressive like behaviors in dams (Salari et al., 2016).

Behavioral Effects in Animal Models

Early studies showed an increased anxiety-like phenotype in pre-natally stressed rats using the elevated plus maze (Fride and Weinstock, 1988). This finding has subsequently been confirmed in several labs using both sexes and multiple strains of both rats and mice (Walf and Frye, 2007; Akatsu et al., 2015; Palacios-García et al., 2015; Zohar et al., 2015). An increase in depressive-like behaviors measured using the forced swim test and sucrose preference test, has also been seen in both sexes and various strains of rat following pre-natal stress (Abe et al., 2007; Butkevich et al., 2011; Guan et al., 2013; Sickmann et al., 2015; Weinstock, 2017). Alterations in social behaviors, spatial memory, novel object recognition, addictive behaviors and sexual activity have also been described following pre-natal stress in rodents (Holson et al., 1995; Markham et al., 2010; Lui et al., 2011; Salomon et al., 2011; Wilson and Terry, 2013; Barzegar et al., 2015; Dong et al., 2018; Gur et al., 2019). Also similar to data in humans, the temporal onset of stress during pregnancy is associated with different cognitive, metabolic and weight outcomes (Mueller and Bale, 2006, 2007). However, despite dramatic advances in technology and methodology in recent times, there has been relatively little novel insight into the mechanistic underpinnings of these behavioral alterations.

SUMMARY AND PERSPECTIVE

A number of excellent reviews (e.g., Monk et al., 2013; Glover, 2014; O'Donnell et al., 2014; Bronson and Bale, 2016) underscore the importance of studies that use model systems in establishing the impact of pre-natal maternal health on offspring development. However, the role for "basic science" studies of pre-natal maternal stress and mental health has remained surprisingly descriptive, with little attention dedicated to the study of biological mechanisms. Despite novel insight from several studies in non-human species (e.g., Gapp et al., 2014; Jašarević et al., 2018; Nugent et al., 2018; Bittle et al., 2019; Jašarević and Bale, 2019), our understanding of candidate mechanisms involved in pre-natal stress remains poor. Furthermore, studies of pre-natal maternal stress in non-human species have largely failed to integrate major advances in the biological sciences, such as single-cell sequencing methods, CRISPR-Cas9 based genetic manipulation and brain organoids. These techniques and systems

are powerful methods for the identification and evaluation of novel mechanisms, which need to be embraced by the field moving forward. Indeed, perhaps the most important research concerning mechanisms for the influence of maternal mental health conditions on offspring neurodevelopment is that which imply mediation by inflammatory signals, which derives almost exclusively from human studies (Hantsoo et al., 2019). Likewise, neuroimaging studies with children have contributed more to our understanding of neural circuits, the development of which is vulnerable to the influence of pre-natal maternal mental health, than has the rather dated neuroanatomical evidence from model systems (see Charil et al., 2010 for a review). We suggest that technological and computational advances in the biological sciences can contribute enormously to our understanding of maternal influences on fetal neurodevelopment. However, an equally important consideration is the need to better align our experimental models of pre-natal maternal stress to the relevant human condition and associated health outcomes. Here, we consider both of these issues for the future of neuroscience research on the topic of pre-natal stress, beginning with the more fundamental issue of alignment.

ALIGNING STUDIES OF PRE-NATAL STRESS IN MODEL SYSTEMS WITH THE HUMAN CONDITION

We suggest that by their very design, the vast majority of research using non-human models best aligns with the maternal trauma stream of human epidemiological research. In these studies, stress is imposed on pregnant females usually at specific time periods over pregnancy. It is common that such studies are expressly designed to address issues related to "critical periods"; when in the course of pregnancy are specific developmental outcomes most likely to be affected by maternal stress? The same issue is often addressed in the analysis of human studies of maternal stress. Our argument is not that the issue of critical periods is irrelevant for studies of maternal influences on neurodevelopment, but rather that the relevance is greater for some models, less so for others. Furthermore, we acknowledge that mechanisms and critical periods identified using existing animal models may also have relevance to instances of more chronic exposures.

The issue of critical periods has a long history in neuroscience and is important for our understanding of the effects of maternal trauma. Such studies may elucidate not only the magnitude of the effect but also the nature of the neurodevelopmental impact. Timing matters. However, this focus has, in our opinion, led to experimental research models that ignore the prevalent human reality of chronic forms of maternal adversity subsumed under our term of maternal distress. The relevant human conditions of "distress" are those of women with high sub-clinical or clinical levels of depression or anxiety and/or chronically elevated perceived stress. In each of these instances, the relevant maternal condition operates throughout the pregnancy. In contrast to the impression engendered by the regrettable term "post-partum depression," the measures that capture the various forms of maternal distress reveal a striking level of stability over the perinatal period. Longitudinal trajectory analyses reveal that depressive symptom levels are largely stable over the pre- and post-natal periods (Santos et al., 2017; Lim et al., 2019) with similar profiles for perceied stress (Lim et al., 2020). Analyses of the ALSPAC cohort reveals modestly higher depressive symptom levels in early pregnancy (Evans et al., 2001; Heron et al., 2004), with a similar trend for symptoms of anxiety (Heron et al., 2004). Latent class analyses and other forms of statistical modeling with longitudinal cohort data show that most women exhibit stable low, moderate or high symptom levels of depression (Cents et al., 2013; Giallo et al., 2014; Van Der Waerden et al., 2015; Park et al., 2018 and see Santos et al., 2017 for review). Only a smaller percentage of women show marked increases in symptom levels that reach clinical levels in the post-partum period, thus revealing a post-partum onset of clinical symptoms. However, there are mixed reports on the stability of anxiety levels during pregnancy and post-partum. Whilst most studies observe lower levels of anxiety after delivery than during pregnancy (Heron et al., 2004; Figueiredo and Conde, 2011), others reveal no difference in anxiety levels (Grant et al., 2008). Therefore, while some women do experience a post-partum onset of depression, the evidence suggests that most women generally report stable levels of depressive and anxiety symptoms over the peripartum period. The trend for perceived stress reveals even stronger evidence for peripartum stability (Lim et al., 2020). In sum, the evidence from longitudinal studies of human mothers reveals a pattern of stability, which implies that the relevant maternal mental health conditions are chronic.

The relative stability of maternal symptoms of depression and anxiety is not surprising since the strongest predictor of post-natal depression is depression during pregnancy (O'Hara and Swain, 1996; Llewellyn et al., 1997; Leigh and Milgrom, 2008). The origins of clinical or high, sub-clinical maternal symptoms of depression and anxiety may extend even further, into the pre-conception period. Marcus et al., 2003 found that almost half of the women with depression during pregnancy had a history of major depressive disorder. Indeed, the most important risk factor for perinatal depression is a prior history of depression (Leigh and Milgrom, 2008; Giallo et al., 2014; Patton et al., 2015). Reviews of psychosocial risk factors for perinatal depression reveal strong evidence for a lack of social support, domestic violence, perceived stress, adverse life events and low socio-economic status (Lancaster et al., 2010; Yim et al., 2015; Biaggi et al., 2016; Santos et al., 2017). These factors are typically stable and also predict levels of depression and anxiety in the general population (Kessler et al., 2003). Stable personality traits such as neuroticism that predict anxiety and depression in the general population, also predict the risk for poor maternal mental health (Leigh and Milgrom, 2008; Martini et al., 2015; Denis and Luminet, 2018). Protective factors such as social support and self-esteem that diminish the risk for depression and anxiety in the general population show a comparable influence on peripartum depression and anxiety (Elsenbruch et al., 2007; Leigh and Milgrom, 2008; Martini et al., 2015).

Since conditions that predict poor maternal mental health in pregnancy pre-date conception, it is unsurprising that so

do levels of depression and anxiety. For many women the relevant maternal condition was present prior to pregnancy and thus predated conception. Perinatal mental health problems are commonly preceded by problems prior to conception, often during adolescence (Patton et al., 2015). Kee et al. (2021) showed that for two-thirds of mothers in a longitudinal cohort with clinical or high, sub-clinical levels of depressive symptoms, the mental health condition was apparent in the pre-conception period. A prospective and transgenerational Australian study provides daunting evidence for the importance of the pre-conceptual period, showing that pre-conception levels of depression predicted emotional reactivity of the offspring independent of maternal depression in either the antenatal or post-natal period (Spry et al., 2020).

IMPLICATIONS FOR STUDIES WITH MODEL SYSTEMS

Studies using model systems inspired by associations observed in humans between the various measures of chronic maternal distress and the risk for affective disorders in the offspring (i.e., "internalizing" forms of psychopathology) must consider the nature of the relevant maternal influence in humans. Longitudinal trajectory analyses clearly show that maternal distress, which predicts an increased risk for depression and anxiety as well as multiple cognitive outcomes in the offspring, is highly stable over the perinatal period. Moreover, antenatal symptoms of depression and anxiety are often consistent with those in the pre-conception period. Thus, the clinically relevant model for the effects of maternal distress on the subsequent risk for depression or anxiety is one that imposes stress on dams over the entire period of pregnancy, including the pre-conception period. Ideally such models would also extend into the post-partum period where the relevant research objectives do not hinge on the timing of effects. Post-partum measures of relevance could involve maternal care, which is affected by pre-natal stress in rodents (Champagne and Meaney, 2006). We acknowledge that this design complicates the issue of timing. However, it is our opinion that these issues can be effectively addressed with more ambitious research designs and discuss this issue later in this paper. The plea is straightforward: research addressing the mechanisms for the association between maternal distress and offspring neurodevelopment and health must respect the chronicity of the relevant maternal conditions in the human condition.

THE PREVALENCE OF MATERNAL DISTRESS

There is a long and impressive scientific literature on the effects of clinical states of maternal depression or anxiety on neurodevelopmental outcomes in the offspring (e.g., Weissman et al., 2006; Monk et al., 2013; Glover, 2014; O'Donnell et al., 2014). Such studies commonly employ case: control analyses of mothers with confirmed clinical diagnoses. These studies document the powerful associations between clinical states of maternal depression and anxiety, and a wide range of neurodevelopmental and health outcomes in the offspring. An obvious issue is whether effects are unique to those women with clinical levels of symptoms. Studies addressing this issue reveal the considerable impact of high, sub-clinical symptom levels of depression (see Meaney, 2018 for review). The psychosocial function and parenting of women with high, sub-clinical levels of depressive symptoms is as affected as are those with clinical levels of depressive symptoms (Judd et al., 1996; Lovejoy et al., 2000; Weinberg et al., 2001). Estimates of the impact of maternal mental health problems based on clinical cut-offs or diagnosis thus underestimate the extent of the problem across the population. In higher income countries, the prevalence of clinical levels of maternal depression is commonly between 10 and 15% (Bennett et al., 2004; Gavin et al., 2005; Wachs et al., 2009; Ertel et al., 2011). The distribution of depressive symptoms across populations is normal and unimodal such that there are 2–3 times more women with high, sub-clinical levels of symptoms compared with those with clinical levels. In economically developed countries, mothers with clinical and high-subclinical levels of depressive symptoms include as much as 40% of the population, thus representing a public health issue of remarkable importance (Meaney, 2018). Moreover, while less well-studied because of the lack of established clinical cut-offs, the prevalence rates of perinatal maternal anxiety appear even higher than those for depression, and consequences for the development of the offspring are at least as impactful as are those of depressive symptoms (van Batenburg-Eddes and Jolles, 2013; Glover, 2014; O'Donnell and Meaney, 2017; Dean et al., 2018; Madigan et al., 2018).

More recent studies underscore the equally pronounced associations between levels of maternal stress and child outcomes (Monk et al., 2013). The influence of these studies has resulted in longitudinal, prospective birth cohort studies with community samples that examine symptoms of depression, anxiety and perceived stress across a continuum. Studies of developmental outcomes in the offspring support a uniform conclusion: The measures of maternal distress operate across a continuum to influence neurodevelopmental outcomes (Meaney, 2018). Neuroimaging as well as measures of cognitive - emotional function in the offspring reveal a graded influence of maternal distress across the normal range of values for measures of maternal distress. Studies of the "unaffected" offspring (i.e., those without depression at the time of testing) of mothers with clinical levels of depression, compared to those of mothers without diagnosed mental disorders, reveal differences in hippocampal and/or amygdala volume, the neural circuitry associated with reward processing and cortical thickness (Chen et al., 2010; Gotlib et al., 2010; Lupien et al., 2011; Peterson and Weissman, 2011). Variation in each of these measures has been observed in community samples that examine symptoms of depression or anxiety across the normal range (Rifkin-Graboi et al., 2013; Qiu et al., 2015; Sandman et al., 2015; Lebel et al., 2016; Wen et al., 2017 and reviewed in O'Donnell and Meaney, 2017; Meaney, 2018). An impressive analysis of cortical thickness (Sandman et al., 2015) yielded a significant association with maternal depressive symptoms despite the almost complete absence of

cases that would pass the clinical screening cut-off for maternal depression. Likewise measures of both socio-emotional and cognitive outcomes in childhood show a graded association with maternal symptoms of depression or anxiety, as well as across levels of perceived stress (Evans et al., 2012; Cents et al., 2013; Yan and Dix, 2016 and see Supplementary Material in Meaney, 2018 for a systematic review). An extensive review (Goodman et al., 2011) reveals that the association between symptoms of maternal depression and internalizing and externalizing problems during childhood is apparent in both community studies as well as in case: control analyses. This spectrum-like association we describe provides strong evidence for causation, which is analogous to the importance of a dose-response relationship in the assignation of causation in toxicology studies.

THE USE OF MODEL SYSTEMS TO STUDY PHENOTYPIC DIMENSIONS

The compelling evidence for the influence of maternal distress across a continuum, as opposed to uniquely clinical conditions, has important implications for studies with model systems. A commonly cited limitation of studies of mental health using model systems is the inability to define disease states in non-human species, and thus establish "face validity." The RDoC (Research Domain Criteria) initiative (Insel et al., 2010; Casey et al., 2013) has mitigated this concern shifting the emphasis from clinically diagnosed syndromes to specific phenotypic dimensions associated with variations in mental health status. This dimensional perspective permits measures to be considered along a continuum applicable to the study of mental health, broadly defined, as opposed to disease. This approach moves the challenge of face validity to that of specific phenotypes, as opposed to complex, multi-dimensional clinical syndromes. Altered patterns of sleep, reward sensitivity, attention, stress reactivity, etc. are features of depressed and/or anxious conditions and disrupted by chronic stress. Each can be assessed in model systems.

Analyses of associations between various measures of maternal distress and child outcomes reveal effects across a continuum, which in our opinion strengthens the case for the use of model systems in which specific maternal phenotypic dimensions can be associated with outcomes in the offspring. One approach that would further strengthen the relevance of studies with non-human species is to relate the stressor to specific features of the maternal condition and outcomes in the offspring. A strength of this approach is that it would allow comparative analyses at the level of specific features of the maternal condition and associations with developmental outcomes in the offspring that would benefit our depth of understanding and inform intervention programs. This approach bears considerable potential to inform intervention studies in humans by identifying the feature of the maternal condition most relevant for the specific offspring outcome. As an example, more fearful female rats exhibit reduced post-partum levels of pup licking/grooming (Champagne and Meaney, 2001) with "downstream" effects on cognitive – emotional outcomes in the offspring (Meaney, 2001). Stress imposed on pregnant, high-licking mothers increases fearfulness eliminating the differences in fear behavior as well as in maternal behavior between high and low pup licking/grooming rats (Champagne and Meaney, 2006). The effect of pre-natal stress on fearfulness mediates the effect on maternal behavior and the subsequent influence on developmental outcomes in the offspring. Environmental enrichment produces exactly the opposite transformation by decreasing fearfulness in low pup-licking females (Champagne and Meaney, 2007). The effect is best understood as an influence of maternal fearfulness and not pre-natal stress, *per se*. Pre-natal stress is the distal influence; maternal fearfulness the proximal, dimensional factor. As noted above, there are multiple functional dimensions that link perceived stress to maternal health in humans that can be approximated in other animals.

MATERNAL DISTRESS: A CONSIDERATION OF MEDIATORS, MODERATORS AND MECHANISMS

Maternal distress during pregnancy confers an increased risk for a psychiatric disorder diagnosis in offspring. The resulting disorder are typically diagnosed in later life with relatively few novel therapeutic options available (Nestler and Hyman, 2010). A better understanding of the pre-natal risk factors and the mechanisms through which they act is likely central to the development of novel therapeutic strategies. To date, mechanistic investigation into the consequences of maternal distress have largely focused on the traditional central mediator of the acute stress response, the hypothalamic-pituitary-adrenal (HPA) axis. This focus has provided valuable insights into how maternal distress can shape psychiatric risk, but it is now clear that alterations within the HPA axis cannot account for the entire spectrum of risk (O'Donnell and Meaney, 2017). We suggest a more diverse investigation of the mechanisms involved in maternal distress, using advanced techniques, is necessary to understand this increased risk. In particular novel -omics approaches [for a full review of -omics based methods and their limitations see Hasin et al. (2017) and Karczewski and Snyder (2018)] offer insight at an unprecedented resolution. Herein, we discuss candidate mediators, moderators and mechanisms, along with the existing evidence for their interaction with maternal distress and psychiatric risk. We make reference to human data where available and draw insight from existing animal models of maternal trauma where appropriate. However, in line with the central thesis of this review, we acknowledge future animal studies of chronic maternal distress are necessary for a comprehensive understanding of the mediators, moderators and mechanisms associated with psychiatric risk.

The HPA (Hypothalamic-Pituitary-Adrenal) Axis

In humans, HPA axis function and stress reactivity in children can be affected by maternal anxiety, depression (O'Donnell et al., 2013; Capron et al., 2015) or maternal trauma (Yong Ping et al., 2015), with alterations in diurnal cortisol rhythms

found to persist until adolescence following pre-natal exposure to maternal anxiety or depression (O'Donnell et al., 2013). In non-human primates (Coe et al., 2003; Murray et al., 2018), guinea pigs (Kapoor and Matthews, 2005, 2008) and rodents (Brunton and Russell, 2010; St-Cyr et al., 2017) exposure to maternal distress has been associated with sex-specific alterations within the HPA axis of the offspring. This effect is also present in several other divergent species, suggesting pre-natal programming of the HPA axis is a highly conserved mechanism (Thayer et al., 2018).

Exogenous administration of synthetic or endogenous glucocorticoids (i.e., dexamethasone and cortisol/corticosterone, respectively), are commonly used to model glucocorticoid excess during pregnancy in a variety of animal models, with exposure associated with a variety of deficits. For instance, pre-natal exposure to dexamethasone in rats produces anxiety-like behaviors in females only (Hiroi et al., 2016), and a reduction in proliferation of neural progenitor cells (Samarasinghe et al., 2011). Using human forebrain organoids expression of genes implicated in psychiatric disorders have also been shown to be affected by dexamethasone exposure (Cruceanu et al., 2020).

However, neither plasma corticotropin-releasing factor (CRF) or hair cortisol are correlated with maternal pregnancy-related anxiety (Kramer et al., 2009; Orta et al., 2018). Salivary cortisol has been associated with anxiety during pregnancy, but only after the 30th week of gestation (Kane et al., 2014) and structural alterations within the fetal brain associated with maternal distress can already be seen by this timepoint (Wu et al., 2020). While in both pregnant and non-pregnant individuals, cortisol is not reliably associated with depression (Seth et al., 2016; Nandam et al., 2020). Therefore, while glucocorticoids demonstrably influence brain development, their contribution to the increased psychiatric risk associated with maternal distress is unclear. Likewise, the follow-up studies of children exposed to therapeutic doses of synthetic glucocorticoids during fetal life has produced equivocal findings using a variety of neurodevelopmental outcome measures (reviewed in O'Donnell and Meaney, 2017).

Allopregnanolone

Allopregnanolone is another secreted steroid (a derivative of progesterone) with well-described signaling functions in the brain (Paul et al., 2020). Maternal plasma concentrations of allopregnanolone increase dramatically during pregnancy (Kancheva et al., 2007) and allopregnanolone is responsive to both acute and chronic stress (Bali and Jaggi, 2014). Pre-natal maternal distress in rats is associated with sex-specific changes in the distribution of allopregnanolone within the offspring brain (Torgersen et al., 2020). Considering allopregnanolone is a strong modulator of GABA (gamma-Aminobutyric acid) signaling (Carver and Reddy, 2013), this may have important implications for circuits throughout the brain.

Allopregnanolone administration can normalize HPA axis reactivity following pre-natal exposure to maternal social stress in female rats (Brunton et al., 2015). Allopregnanolone is also decreased in the brain of individuals with depression (Uzunova et al., 1998; Schüle et al., 2006), as well as in non-human animal models of depression (Almeida et al., 2020). However, a study of 284 pregnant women did not find an association between plasma allopregnanolone and depression at the 18th week of gestation (Hellgren et al., 2017). Although, another study found that allopregnanolone levels measured in the second trimester were associated with an increased risk of post-natal depression (Osborne et al., 2017). In line with the RDoC initiative outlined earlier, allopregnanolone should be characterized with respect to specific symptoms and imaging phenotypes to clarify its role in maternal distress. Nevertheless, the protective effects of allopregnanolone in non-human models following pre-natal stress (Brunton et al., 2015) warrant further investigation.

Resiliency

A large proportion of individuals exposed to pre-natal maternal distress do not develop a psychiatric disorder. This concept of resiliency is more commonly studied in non-pregnant adult animals (Russo et al., 2012) but it is unclear how well these findings relate to pregnancy considering the distinct social and physiological environments involved. Moreover, there are a complex set of interactions between maternal genetics and environment, and subsequently fetal genetics and environment which need to be considered. To understand psychiatric risk in offspring, the nature of resilience at each of these levels must be characterized. For instance, maternal resilience to the psychiatric correlates of chronic distress, may be mediated through genetic mechanisms. This does not preclude elevations in blood borne signaling molecules associated with distress which may entrain the fetal brain, thereby maintaining fetal exposure and elevating fetal risk in the absence of maternal symptoms of distress. Moreover, there are an increasing number of loci associated with resilience (Maul et al., 2020), underlining the importance of understanding the polygenic component of resilience and its associated mechanisms. It should also be noted that resiliency is multi-factorial in nature and also influenced by maternal behaviors (Bolten et al., 2012; Brotnow et al., 2015). Further, pre-natal stress can affect subsequent responses to the post-natal environment through plasticity related mechanisms (Hartman and Belsky, 2018).

Polygenic Risk

A major difficulty in human studies which assess psychopathology risk following instances of maternal trauma or distress is distinguishing the relative contribution of genetic and environmental components. The increasing size of human cohorts and genetic analyses have given clear demonstrations of the polygenic risk associated with psychiatric disorders. These studies also indicate large degrees of genetic pleiotropy and indicate relevant genes are highly expressed during fetal development (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013, 2019). Furthermore, this polygenic component can interact with the environment to modulate outcome. For instance, Qiu et al. (2017) reported that a polygenic risk score for major depressive disorder moderated the association between pre-natal maternal symptoms of depression and hippocampal and amygdala volume assessed with neonatal magnetic resonance imaging (MRI). The interaction between this polygenic component and the environment is key to

understanding absolute risk, but this polygenic risk cannot currently be accurately modeled in non-human models. In recent years, brain organoid models have become a reproducible and accessible human model of early neurodevelopmental processes (Camp et al., 2015; Yoon et al., 2019). They provide an excellent opportunity to model the interaction between polygenic risk and environmental perturbations or signaling mediators, on relevant neurodevelopmental processes.

Epigenetics

Epigenetic marks, such as DNA methylation and histone modifications, are also important factors to consider. They are dynamically present throughout neurodevelopment (Spiers et al., 2015; Amiri et al., 2018) and likely have a role in determining psychiatric outcomes (Kuehner et al., 2019). Sexual diversification during brain development also heavily relies on epigenetic mechanisms (McCarthy et al., 2017) and as such may contribute to sex-related differences in psychopathology following maternal distress. In humans, changes in DNA methylation within cord blood and blood during childhood have been associated with pre-natal maternal distress (Cao-Lei et al., 2014; Wu et al., 2018). Similarly, animal models of maternal distress have demonstrated changes in DNA methylation and histone modifications in the placenta and fetal brain (Peña et al., 2012; Matrisciano et al., 2013; Benoit et al., 2015; Nugent et al., 2018).

Much of this work has been carried out using candidate methods but epigenetic alterations at a single locus are unlikely to offer robust and novel mechanistic insight. Genome wide approaches are now generally affordable with well-described analysis pipelines (Yong et al., 2016; Cuvier and Fierz, 2017) and should be further embraced to generate novel insight. Furthermore, most of this work has been correlational in nature due the lack of tools to specifically modify the epigenome. Recently with the revolution in CRISPR-Cas9 genome editing technology, a range of epigenetic modifying tools have become available (see Yim et al., 2020 for review). These tools allow for the specific manipulation of an epigenetic mark, to identify causal mechanisms for relevant psychiatric phenotypes. These methods will be central for future studies to examine the ability of epigenetics to mediate outcomes following maternal distress.

Circadian Rhythmicity

Circadian rhythms are present in biology from the behavioral (i.e., sleep-wake cycles) down to even the epigenetic level (Etchegaray et al., 2003; Azzi et al., 2014), and changes in circadian rhythmicity are a central component of depression and anxiety disorders (Germain and Kupfer, 2008; Walker et al., 2020). Circadian disruption during pregnancy in the form of a fixed night shift schedule is associated with an increase in fetal loss during the late stages of gestation (Zhu et al., 2004) and other adverse pregnancy outcomes (Cai et al., 2019). In animal models, constant light is associated with altered gestational length (Mendez et al., 2016; Gatford et al., 2019), along with altered circadian rhythms (Ohta et al., 2006) and spatial memory (Vilches et al., 2014) in offspring. It is not clear whether this effect is moderated by maternal psychological distress, but gestational restraint stress in mice alters circadian gene expression in the suprachiasmatic nucleus of offspring (Yun et al., 2020) and pre-natal maternal anxiety in humans is associated with sleep problems in offspring at 18 and 30 months (O'Connor et al., 2007).

Mutation of the core circadian regulators *Clock* or *Per1* and *Per2* in mice results in anxiety-like behaviors (Roybal et al., 2007; Spencer et al., 2013). *Clock* is involved in the regulation of critical periods during neural circuit maturation (Kobayashi et al., 2015) and *Per1* is a well-characterized stress response gene (Al-Safadi et al., 2015). However, the function of circadian patterns of gene expression during typical brain development requires further study in order to establish their role in anxiety- and depressive-like behaviors. The development of novel single cell analysis methods and genetic models (Fonseca Costa et al., 2020; Shan et al., 2020; Wen et al., 2020) offer the opportunity to study these effects at unprecedented resolution and will likely play a central role in these future investigations.

Furthermore, several key maternal hormones are secreted in circadian patterns. For instance melatonin, which can readily cross the placenta (Naitoh et al., 1998) and has neuro-protective effects in humans and animal models following inflammation and asphyxiation (Fulia et al., 2001; Welin et al., 2007; Castillo-Melendez et al., 2017; Aridas et al., 2018). The melatonin receptor is expressed in many nuclei throughout the fetal brain including many within the hypothalamus (Thomas et al., 2002) and circulating nocturnal melatonin is decreased during pregnancy but increased in the post-partum period in women with depression (Parry et al., 2008). Interestingly, maternal melatonin can modulate fetal cortisol levels in non-human primates (Torres-Farfan et al., 2004), but the dynamics of melatonin signaling in the fetal brain during maternal distress and their consequences for subsequent outcomes are largely unknown. Many key hormones of the HPA (hypothalamic-pituitary-adrenal) axis are also secreted in a circadian fashion, with changes seen in their secretion in offspring exposed to maternal distress (O'Donnell et al., 2013).

Extracellular Vesicles (ECVs)

Another form of maternal-fetal communication occurs through ECVs, which are small membrane enclosed bodies which all cells are capable of producing. They can contain nucleic acids, proteins or metabolites and play an important role in cell-cell signaling in a number of contexts, including maternal-fetal signaling (Van Niel et al., 2018; Buca et al., 2020). ECVs are involved in signaling pathways associated with depression (Brites et al., 2015) and can affect neurodevelopment in offspring following paternal distress through pre-conception signaling mechanisms in sperm (Chan et al., 2020). However, the role of ECVs in maternal-fetal signaling during maternal distress remains largely unknown. Deep RNA sequencing and global proteomic approaches enable an unbiased analysis of ECV cargo in both humans and animal models (Huang et al., 2013; Choi et al., 2015) and these techniques will be important in establishing the role of ECVs in maternal-fetal signaling during distress. This is particularly true for processes, such as inflammation, which

have frequently been shown to use ECV signaling mechanisms (Słomka et al., 2018; Van Niel et al., 2018).

Inflammation

Both human and non-human studies have described a central role of inflammatory mechanisms in depression (Slavich and Irwin, 2014; Miller and Raison, 2016). A wealth of data also indicate inflammatory mechanisms may, at least in part, mediate the effects of maternal distress on the fetus (see Hantsoo et al., 2019 for review). Considering perinatal inflammation is a potent modifier of neurodevelopment and offspring behavior (Hagberg et al., 2015; Choi et al., 2016; Reed et al., 2019), the role of inflammatory mechanisms in this capacity have been understudied.

Maternal distress in humans has been associated with altered levels of plasma inflammatory mediators throughout pregnancy (Coussons-Read et al., 2007; Blackmore et al., 2011; Walsh et al., 2016; Gilman et al., 2017) and in rodents pre-natal stress is associated with elevated cytokines and altered microglial phenotypes in the neonatal brain (Diz-Chaves et al., 2012; Slusarczyk et al., 2015; Chen et al., 2020). In adult mice, peripheral immune cells can regulate anxiety-like behaviors through IL-17 (Alves de Lima et al., 2020) and maternal IL-17 has been shown separately to modulate neurodevelopment and social behavior in mice exposed to an inflammatory stimulus (Choi et al., 2016; Reed et al., 2019). Furthermore, in mice IL-6 has been shown to drive phenotypic alterations in microglia within the embryonic cortical plate following pre-natal stress (Gumusoglu et al., 2017). Intriguingly, research suggests that pre-natal administration of non-steroidal anti-inflammatory drugs can ameliorate some of the symptoms associated with a pre-natal CVS model (Bronson and Bale, 2014). Understanding the mediators and signaling pathways involved in these effects are important considerations for future studies. Recently, large reference datasets for the automatic annotation of peripheral immune cells following single cell sequencing (Hao et al., 2020) have been developed, which could be used in future studies alongside global proteomic approaches in both the maternal and fetal compartments to identify maternal signaling mediators involved in the conferral of risk for psychopathology.

Placental Dysfunction

Considering the central role of the placenta in maternal-fetal exchange, it is a prime candidate to mediate the *in-utero* effects of maternal distress including those from inflammatory mediators. However, it is surprisingly understudied in this context, despite insightful results in animal models. For instance, in a pre-natal CVS model, placental expression of *O-GlcNAc transferase* (*OGT*) has been described as a key moderator of fetal growth and HPA axis functionality (Howerton and Bale, 2014) and placental H3K27me3 has been shown to be involved in establishing sex specific responses to CVS (Nugent et al., 2018). The enzyme 11β-HSD2 which converts glucocorticoids to their active or inactive form at the placenta, thereby controlling fetal glucocorticoid exposure, is also affect by maternal anxiety in humans (O'Donnell et al., 2012).

The placenta is unique in that it can be sampled and examined using -omics methodologies with little physiological intrusion to the mother or child. Preliminary studies have taken advantage of this by using arrays to identify gene expression changes within the placenta associated with maternal depression (Olivier et al., 2015; Edvinsson et al., 2019). The more recent approach of single cell sequencing has been used in human placenta tissue to identify changes associated with preeclampsia and preterm birth (Tsang et al., 2017; Pique-Regi et al., 2019), and a similar approach to delineate cell specific gene expression and epigenetic states in the placenta of pregnancies associated with anxiety or depression would be extremely informative.

Microbiome

Similarly to the placenta the microbiota can be examined in both the mother and fetus with relatively little intrusion and in recent years the sensitivity of the microbiome to the environment and its importance in brain development has become appreciated. For instance, in adult animals the microbiome mediates the behavioral effects of stress (Marin et al., 2017; Pearson-Leary et al., 2020) and in humans, probiotics during pregnancy have proven effective in alleviating post-natal symptoms of depression and anxiety in mothers (Barthow et al., 2016; Slykerman et al., 2017). This is particularly pertinent as the maternal microbiome is a crucial mediator of typical fetal brain development (Vuong et al., 2020). In rodents, pre-natal stress can drive changes in offspring microbiota composition and the intestinal transcriptome (Jašarević et al., 2018), while in humans microbiota changes within the meconium have been associated with maternal anxiety (Hu et al., 2019). The dynamics of these changes and their involvement in mediating psychiatric risk will be important future considerations. In recent proof-of-concept work, fetal microbiota composition was demonstrated to be amenable to manipulation through fecal microbiota transplantation (Korpela et al., 2020), which may point to a novel therapeutic strategy if microbiota are demonstrated to mediate psychiatric risk associated with pre-natal exposure to maternal distress.

Neurogenesis and Neuronal Maturation

A wealth of human MRI data points to both structural and functional changes within the brain of children following maternal distress (see Lautarescu et al., 2020a for review). Structural and micro-structural changes have also been described in the neonatal period (Li et al., 2012; Rifkin-Graboi et al., 2013; Qiu et al., 2015; Lautarescu et al., 2020b) and *in utero* (Wu et al., 2020). Processes such as neurogenesis and gliogenesis are obvious candidates to mediate these early changes, and indeed a variety of models, across different species and brain regions, have demonstrated altered neurogenesis (Coe et al., 2003; Kraszpulski et al., 2006; Rayen et al., 2011) and dendritic spine distribution (Murmu et al., 2006; Bock et al., 2011; Petit et al., 2015) in the neonatal and adolescent period following pre-natal maternal distress. Interneuron maturation and migration is also affected by pre-natal maternal distress in rodents (Fine et al., 2014; Lussier and Stevens, 2016), which may be of particular interest considering the expanded program of interneuron generation

and migration seen specifically in humans (Paredes et al., 2016). As such, changes in neurogenesis and neuronal maturation are candidates to underlie the structural alterations seen in humans following maternal distress, but further work is needed to understand the signaling pathways and cellular dynamics associated with these effects. Furthermore, very few studies have leveraged MRI techniques to investigate changes associated with maternal distress in model systems, despite the widespread availability of the hardware required for image acquisition. Considering the routine use of MRI in human studies, MRI studies with mammalian model systems would provide an elegant comparison without the inevitable confounders associated with human studies.

CONCLUSION

In this review we highlight important considerations for the future of pre-natal stress research. We indicate that the majority of pre-clinical work aligns with instances of maternal trauma and, while acknowledging the importance of this work, we suggest the prevalence of anxiety and depressive disorders amongst pregnant women dictates a higher priority within non-human models. We suggest existing models of maternal distress, such as the chronic variable stress model, should be expanded to all of gestation and the pre-conception period, a timeline which is analogous to the relevant human risk factors. Considering the limited novel mechanistic insights into the underpinnings of psychiatric risk, we also suggest a more diverse range of mechanisms need to be investigated using advanced techniques and analysis methods.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EF and MM prepared the manuscript. CP and MK provided substantial editing and conceptual input. All authors contributed to the article and approved the submitted version.

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Trajectories of Mother-Infant Communication: An Experiential Measure of the Impacts of Early Life Adversity

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Caretaking stability in the early life environment supports neurobehavioral development, while instability and neglect constitute adverse environments that can alter maturational processes. Research in humans suggests that different types of early life adversity (ELA) can have differential effects on caretaker relationships and later cognitive and social development; however, identifying mechanistic underpinnings will require animal models with translational validity. Two common rodent models, maternal separation (MS) and limited bedding (LB), influence the mother-infant relationship during a critical window of development. We hypothesized that these paradigms may affect the development of communication strategies on the part of the pup. Ultrasonic vocalizations (USVs) are a care-eliciting mechanism and ethologically relevant response to stressors in the rat pup. USV emission rates and acoustic parameters change throughout early development, presenting the opportunity to define developmental milestones in USVs that would reflect neurobehavioral aberrations if disrupted. This study investigated the effects of MS or LB on the dam-pup relationship by quantifying pup USVs, maternal behavior, and the relationship between the two. First, we used a generalized additive model approach to establish typical developmental trajectories of USV acoustic properties and determine windows of change in MS or LB rearing. Additionally, we quantified maternal behaviors and the predictability of maternal care sequences using an entropy rate calculation. MS and LB each shifted the developmental trajectories of USV acoustic parameters and call types in a sex-specific manner. MS more often impacted male USVs, while LB impacted female USVs. MS dams spent more time passive nursing, and LB dams spent more time on the nest. The predictability of maternal care was associated with the rate of USV emissions exclusively in females. Taken together, findings demonstrate sex- and model-specific effects of rearing environments on a novel developmental trajectory involving the mother-infant relationship, facilitating the translation of animal ELA paradigms to assess later-life consequences.

Keywords: early life adversity, maternal separation, limited bedding, development, ultrasonic vocalizations, rat

INTRODUCTION

The nature of the parent-offspring relationship is a particularly critical component of the early postnatal environment in mammals. In humans, quality of parental care has been shown to be predictive of adolescent and adult psychopathologies, contributing to variations in stress and anxiety regulation (McGoron et al., 2012; Kundakovic and Champagne, 2015). For example, parental abuse and neglect, including cases of severe deprivation in institutionalized infants, is associated with increased rates of autistic-like traits, personality and anxiety disorders, and depression (Trickett and McBride-Chang, 1995; Johnson et al., 1999; Rutter et al., 1999; Enoch, 2011). Unsurprisingly, the long-term outcomes of early life adversity (ELA) have been widely studied in humans, non-human primates, and rodents. Such research corroborates that the neonatal window defines a critical period wherein experiences shape developmental trajectories.

As clinical observations dating back to Erikson (1959) have shown, developmental milestones marking critical periods for affective and cognitive maturation during early life can predict later function. An example of a specific predictor of later cognitive or emotion-regulation deficits include failure to meet language development milestones by 24 months (Peyre et al., 2017). Since the timing and sequential pattern of milestone acquisition form an important marker of neurological integrity, these measures can provide crucial information regarding individualistic disturbances attributable to early adversity. Traditionally, animal models have allowed the investigation of mechanisms underpinning effects of adversity on later-life behaviors, proving to be useful when parsing effects of specific experiential domains on development. However, to date, the evidence associating discrete facets of ELA with developmental milestone achievement in the context of socio-emotional development are lacking.

Caretaker behavior is crucial to mammalian development, regulating physiological homeostasis and suppressing the adrenocortical response during the preweaning period (Sapolsky and Meaney, 1986; Hofer, 1996; Champagne et al., 2001; Zimmerberg et al., 2003). Thus, rodent models of ELA typically employ methods to disrupt dam-infant interactions, leading to long-term effects in offspring. In one such model, maternal separation (MS), pups are separated from the dam for a specified time daily during the preweaning period. MS results in changes in the hypothalamic-pituitary adrenal (HPA)-axis stress response, immune function, neuronal morphology, and functional connectivity in the prefrontal cortex and connected regions (Lehmann et al., 2002; Aisa et al., 2007; Enoch, 2011; Chocyk et al., 2013; Wieck et al., 2013; Holland et al., 2014). These changes occur alongside behavioral aberrations such as increased anxiety-like behavior and social deficits (Monroy et al., 2010; Holland et al., 2014; Ganguly et al., 2015). While MS inherently reduces the amount of time the pups spend in the dam's presence, there is also evidence that dams engage in compensatory care after being reunited with pups (Huot et al., 2004). Partially to limit this compensatory behavior and interactions with experimenters, the limited bedding and nesting model (LB) was developed. LB disturbs normal rearing by providing inadequate nesting material in the cage, which, in turn, influences maternal behavior (Gilles et al., 1996; Brunson et al., 2005; Walker et al., 2017). In this paradigm, the dam's basal corticosterone levels increase, leading to fragmented, unpredictable, and abusive care patterns (Molet et al., 2016; Walker et al., 2017; Gallo et al., 2019). Pups reared under LB conditions likewise show deviations in HPA axis regulation in addition to later-life neuronal dysfunction, anhedonia, cognitive deficits, and anxiety-like behavior (Brunson et al., 2005; Bath et al., 2016; Molet et al., 2016).

Data from both the human and animal literature have found certain outcomes associated with ELA to be sex-specific (MacMillan et al., 2001; Dickie and Armony, 2008; Bath et al., 2016; Grassi-Oliveira et al., 2016; Bondar et al., 2018). In rodents, this effect may be partially attributable to male and female pups receiving different levels of caregiving behaviors. For example, dams preferentially direct anogenital licking to male pups (Richmond and Sachs, 1983). Variations in this behavior regulate epigenetic modifications contributing to sexually dimorphic development of the stress and reproductive systems (Champagne and Meaney, 2001; Renard et al., 2007; Roth et al., 2009). The specific factors of adverse rearing environments that mediate sex-dependent pup-directed behaviors have not been widely explored.

Dam-pup interactions are facilitated in part by auditory signals in the form of isolation-induced ultrasonic vocalizations (USVs) (Muller et al., 2010). Recent research supports that USVs are an indicator of affective state and elicit maternal care behaviors from the dam, not simply a byproduct of general arousal (Muller et al., 2010). Thus, from an evolutionary standpoint, vocalizations are critical for pup survival (Branchi et al., 2001; Ehret, 2005; Wöhr and Schwarting, 2008; Lingle et al., 2012). USVs undergo developmental changes during the preweaning period, with call duration, peak frequency, and bandwidth increasing from P10 to P17 (Brudzynski et al., 1999). Further, male pups emit USVs at a greater rate and a lower frequency than females (Bowers et al., 2013), but developmental trajectories for USVs have not been clearly defined in both sexes. If these acoustic parameters contribute to sex-specific caregiving patterns and carry functional significance in neurobehavioral development, USVs in growing pups could be interpreted as developmental milestones, where shifts or delays indicate atypical development (Grimsley et al., 2011). Standard milestones of early development include physical and reflexive measures such as eye opening, fur development, and the mid-air righting reflex, which typically appear between P10 and P20 (Heyser, 2004). Notably, these physical milestones are impacted by both MS and LB in a sex-dependent manner (Demaestri et al., 2020). Including an ethologically relevant measure of affective state, such as infant USVs, will contribute to the translational validity of rodent models of ELA.

The current experiments sought to define developmental trajectories for acoustic parameters of USVs in male and female rats in order to assess the pups' affective response to adversity over time. Firstly, we built a model of the typical development

of USV parameters. These include standard acoustic measures in addition to analysis by the USV's sonographic structure or call type, which have been hypothesized to play a role in dam-pup communication (Brudzynski, 2005). The MS and LB models of ELA were implemented to determine their individual effects on the model of typical USV development. We assessed each paradigm's effects on the model USV curves to identify critical periods when vocalizations were susceptible to environmental changes. The second goal of the study was to determine how rearing condition influenced maternal behaviors. Frequency and duration of tactile stimulation, via grooming and engaged nursing is known to support pup development (Champagne et al., 2001). In addition, there is growing evidence of the importance of the temporal predictability of maternal care behavior in regulating neurobehavioral outcomes (Walker et al., 2017). Thus, we assessed individual rat dam behaviors and the predictability of behavioral sequences by calculating the entropy of behavioral transitions. Finally, to investigate the degree to which maternal care was associated with changes in the pups' characteristics, we assessed whether maternal behaviors covaried with differences in USV emissions between rearing conditions. These studies were aimed to elucidate individual differences in dam-pup interactions that contribute to the dysfunctional behavioral development in two widely used models of ELA.

MATERIALS AND METHODS

Subjects

All experiments were performed in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH) with approval from the Institutional Animal Care and Use Committee at Northeastern University. Male and female Sprague-Dawley rats originally obtained from Charles River Laboratories (Wilmington, MA) were used to breed subjects for this study. One male and one female were caged together until pregnancy was confirmed, a maximum of 4 days. All females were primiparous, and pregnant dams were housed singly. Parturition was checked daily, and the day of birth was denoted as P0. On P1, litters were culled to 10 pups, maintaining 5 males and 5 females wherever possible. Whole litters were randomly assigned to be reared under control (Con), MS, or LB paradigm. To avoid litter effects, a maximum of two males and two females were used from each litter (n = 10/group). Dams and pups in Con rearing were housed under standard laboratory conditions in polycarbonate wire-top caged with a 2–3 inch layer of pine shave bedding covering the bottom of the cage. Food and water were available *ad libitum*. The facility was kept on a 12-h light/dark cycle (light period between 0700 and 1900) with regulated temperature (22–23°C) and humidity (37–53%). Con pups were left undisturbed except to be weighed on P9 and P20, and USV recording days on P5, P10, P15, and P21 (**Figure 1**). Pups were identified by toe clips performed on P5 after USVs were recorded.

Maternal Separation

From P2-20, MS pups were separated from their dams and littermates for 4 hours per day (0900–1300 h) as previously described (Holland et al., 2014; Grassi-Oliveira et al., 2016; Ganguly et al., 2019). MS dams remained in their home cage in a separate room. From P2-10, separations took place in individual plastic cups with home cage pine shavings and droppings in a circulating water bath kept to 37°C to maintain nest odor and temperature. From P11-20, when pups are able to thermoregulate, they were individually separated in small mouse cages with the same home cage pine shavings. Pups were returned to dams in standard laboratory cage conditions identical to Con.

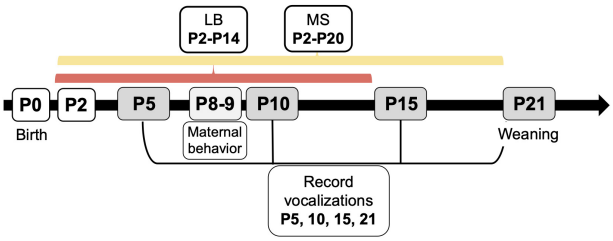

FIGURE 1 | Experimental timeline for early life stress paradigms, ultrasonic vocalization recordings, and maternal behavior recordings. Whole litters were assigned to control (Con), maternal separation (MS), or limited bedding (LB) groups at birth. On P2, MS litters began daily separations from dam and littermates until P20, and LB litters began housing in standard cages with a wire mesh floor and one-half of a paper towel provided for nesting material until P14. Con litters were reared in standard conditions until weaning. Ultrasonic vocalizations (USVs) were recorded on P5, 10, 15, and 21. Maternal behavior was monitored from P8-P9.

Limited Bedding

On P2, LB dams and litters were transferred to a cage with thin layer of pine shaving bedding placed under a wire grid (0.25″ × 0.25″ grid size) resting 0.5″ off the cage floor (Molet et al., 2014; Walker et al., 2017). A small handful of bedding and half of a C-fold paper towel were placed on top of the wire mesh for nesting material. This arrangement allows waste to fall beneath the wire grid without contributing to nesting material in the cage. LB housing took place until P14. Therefore, LB pups experienced a different total amount of time in the adversity rearing condition than MS pups. These timelines were chosen because of their standardization as accepted timelines in previous literature. Additionally, the purpose of the study was not to make direct comparisons between the two paradigms, but to examine the responses compared to the control group. For this study, MS and LB pups may be considered to have received the same "dose" of adversity for all outcome measures with the exception of USVs on P15 and P21.

USV Recording and Classification

USVs were recorded for the same pup (identified by toe clips) on P5, 10, 15, and 21. Pups from each rearing condition were recorded in the same manner. Each pup was isolated for 5 min while audio was recorded with an ultrasonic microphone (Avisoft Bioacoustics, model CM16/CMPA) positioned 10 cm above the cage. On P5 and 10, pups were recorded in individual plastic cups with home cage pine shavings in the water bath kept at 37°C. Since cold temperatures and newly changed bedding have

been shown to induce USVs, pups were kept at a thermoneutral temperature to obtain a naturalistic simulation of isolation in a familiar environment. On P15 and P21, pups were recorded in small mouse cages with home cage bedding. Following the recording, the pup was returned to the home cage.

Audio files were uploaded and analyzed using Deepsqueak (Coffey et al., 2019), a Matlab program for USV analysis. USVs were visualized by converting each file to spectrograms using Fast Fourier Transformation (FFT). Each call detected by Deepsqueak was manually confirmed or rejected by a trained experimenter. Deepsqueak uses contour detection to generate call statistics from the spectrograms of accepted calls and accurately separates USVs from broad spectrum noise. The duration, maximum frequency, minimum frequency, and peak frequency of each call are extracted from the contour. To cluster USVs by sonographic structure using the shape, frequency, and duration from the contours, a previous dataset of 12,227 USVs from 75 subjects was used to train a supervised classifier in Deepsqueak. USV types were developed based on pup calls identified by Brudzynski et al. (1999). The types identified in manual classification of the training dataset were complex, constant, downsweep, fragmented, inverted-U or upright-U, upsweep, and trill (call type criteria outlined in **Table 1**; representative images in **Figure 2**). In the supervised classification, trills and complex calls were combined into one category due to high confusion matrix correlations between the two types.

Maternal Behavior Assessment

Beginning on P8, home cage recordings were taken at three 30-min time-points (at 1430 and 2330 on P8 and 0830 on P9) for later assessment of maternal behavior. A red light was used during the dark phase recording (2330). One time point during the dark phase (2330) was chosen to account for changes in maternal behavior due to nocturnality (Capone et al., 2005). An experimenter blind to experimental condition coded the videos continuously using EthoVision's manual scoring feature to acquire total durations the dam engaged in each behavior assessed. Dams were identified as engaging in one of the following behavior states: on nest, off nest, arched-back nursing (ABN), passive nursing, licking and grooming (LG), licking and grooming while nursing, and self-grooming (Capone et al., 2005). The dam was considered on the nest when she was in the same cage quadrant as the nest. ABN was scored if the dam was positioned over the nest with legs in a wide stance and an arch or curved back that is repeatedly initiated. Passive nursing was scored when pups were latched and nursing, but the dam is unengaged, sleeping, or laying down. LG was scored when the dam licked one or more pups. LG occurring during a nursing bout was scored separately. We added LG with and without nursing for the final calculation of LG duration. Self-grooming was scored when the dam licked herself or cleaned her face. Scoring reliability of above Cohen's kappa = 0.9 was established between two experimenters using Cohen's kappa reliability testing.

Statistical Analyses

USVs

Statistics were calculated in R 3.6.2 (R Core Team, 2019). Generalized additive models (GAMs) were used to assess the effects of age on USV parameters. GAMs are predictive models that incorporate a smooth function of one or more covariates to model non-linear relationships without assuming the shape of the model *a priori*. The *mcgv* (Wood, 2003) package was used to specify the model according to the following form:

$$y_{ij} = \alpha_0 + \alpha_1 Rearing_{ij} + f_j(Age_i) + \varepsilon_i$$

where α_0 is the intercept, α_1 is the difference between the mean response in the j^{th} rearing condition and α_0, f_j(Age) is the smooth function representing the relationship with age for each rearing condition, and ε_i is the residual (Rose et al., 2012). The smooth function was constructed with four knots and a "by" variable, which allows the smoothing of age to interact with rearing condition as a factor and thus incorporates separate age-dependent response curves for each rearing condition into a single model. Periods of age-dependent change were identified using the gratia (Simpson and Singmann, 2020) package to perform a posterior simulation of 10,000 iterations and calculate the first derivative of their estimated smooths using finite differences. Derivatives and their 95% confidence intervals were computed from a multivariate normal distribution fitted to the model parameters. Ages where confidence intervals did not include zero ($p < 0.05$) were identified as periods of age-related change.

In the first set of analyses, GAMs were performed for males and females separately. All rearing conditions were included in the model, but MS and LB were independently compared to Con. To determine age periods where MS or LB models differed from Con, prediction matrices of fitted values of the responses were created with the GAM as specified above. Then, rows of the new matrix corresponding to MS or LB were subtracted from

TABLE 1 | USV call type descriptions for manually classified calls that were used to train the supervised classifier.

Call Type	Criteria
Complex	At least 3 changes in frequency sweeps
Constant	0 changes in frequency sweeps
Downsweep	Continuous downward slope
Fragmented	At least 1 frequency jump fluid in time
Inverted-U or Upright-U	1 frequency change Upward to downward or downward to upward
Trill	Rapid frequency fluctuations over large bandwidth (>3 changes in frequency; bandwidth > 8 kHz)
Upsweep	Continuous upward slope

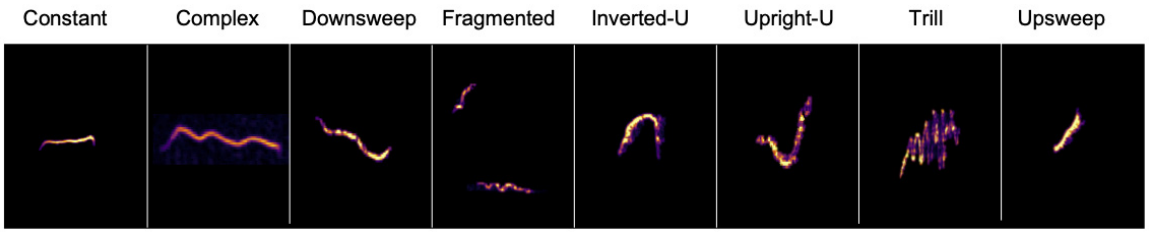

FIGURE 2 | Representative spectrograms of USV call types.

those corresponding to Con, and standard errors were calculated to generate 95% confidence intervals of the difference between rearing conditions (Rose et al., 2012). Significant differences between Con and MS or LB were identified as ages where 95% confidence intervals did not include zero ($p < 0.05$). A second analysis was performed to determine the effects of sex within each rearing condition. We determined age-related changes using a GAM defined by the following model:

$$y_{ij} = \alpha_0 + \alpha_1 Sex_{ij} + f_j(Age_i) + \varepsilon_i$$

Differences between males and females were determined using the methods described above.

Maternal Behavior Group Comparisons

Maternal behavior on P8-9 was directly compared between paradigms, as at this time the differing length of adversity paradigms was not relevant. Maternal behavior analyses were conducted using the *rstatix* (Kassambara, 2020) package in R. Data were assessed for normality of residuals using the Shapiro-Wilk Test and homogeneity of variances using Levene's Test. One-way ANOVAs were conducted to compare the total durations and frequencies of maternal behaviors between rearing groups at each time point. Significant ANOVAs were followed by Tukey's HSD *post-hoc* multiple comparisons tests to determine differences between each rearing condition. One Con litter and three MS litters were excluded from analysis at the 2330 time point due to technical failure of the red light, making videos unviewable. Results were visualized using GraphPad Prism 7.

Entropy Analysis

Entropy scores were calculated for each dam's individual transition matrix according to the formula:

$$H(\chi) = -\sum_{ij} \mu_i P_{ij} \log P_{ij}$$

where P_{ij} is the probability of transitioning from behavior i to behavior j, and m_i is the stationary distribution. Because some probability matrices were singular and thus non-invertible, the stationary distribution was estimated with the generalized inverse (*ginv*) function in R.

Analyses of covariance (ANCOVA) were calculated to test for any covariance of pup weight with maternal entropy scores (at P8-9) or total number of USVs (at P21). ANCOVA were also calculated to determine the effect of rearing on the total number of USVs emitted at each age while adjusting for maternal entropy scores at each time point analyzed. Results were evaluated for statistical significance using a bonferroni-corrected *p*-value of 0.0127 to account for multiple comparisons. All statistics were carried out in R using the *rstatix* (Kassambara, 2020), *emmeans* (Lenth et al., 2020), and *lsr* (Navarro, 2015) packages to assess normality of residuals using Shapiro-Wilk Test, homogeneity of variances using Levene's Test, and effect sizes (h^2), and results were visualized using *ggplot2* (Wickham et al., 2020). Observations with standardized residuals greater than 3 were identified as outliers and removed from analysis.

RESULTS

USV Acoustic Properties

Statistics for USV acoustic properties (total calls, average peak frequency, and average bandwidth) compared between rearing conditions within sex can be found in **Table 2**. Statistics for USVs by call type can be found in **Table 3**. In order to reveal sex effects, statistics directly comparing male and female USV acoustic properties within each rearing condition can be found in **Supplementary Table 1**.

Total USVs

To ensure that total USV rate was not attributable to variability in pup weight, analyses of covariance (ANCOVAs) were conducted for P20 total USV number with pup weight at P20 as a covariate. Pup weight was not found to covary with USV emissions ($p > 0.05$ for both males and females; **Supplementary Table 2**).

Analysis of generalized additive model fits of total USV emissions indicate that in males, all rearing groups follow a developmental trajectory dependent on age (approximate significance of smooth terms (SST): Con: $F = 3.459$, $p = 0.019^{*}$; MS: $F = 10.264$, $p < 0.001^{***}$; LB: $F = 4.778$, $p = 0.003^{**}$; **Figure 3A**). 95% confidence intervals of model derivatives identified periods of significant age-related change in Con (increasing P5-11.27), MS (increasing P5-9.82 and decreasing P12.72-21), and LB (increasing P8.86-15.05 and decreasing P18.17-21). Differences between the prediction curves demonstrated that MS pups emitted more calls than Con between P6.45 and P12.16, but fewer calls between P17.3 and P21.

In females, MS and LB groups demonstrated significant age-related change in total USV number, while Con animals demonstrated a trending, but non-significant age-related change

TABLE 2 | Summary statistics of USV acoustic properties analyzed by generalized additive models comparing rearing conditions within each sex.

			edf	Ref.df	F	*p*-value	Periods of age-related changes	Age periods significantly different from Con
Total USVs (#)	Males	Con	2.141	2.511	3.459	0.01889*	5–11.27 (+)	
		MS	2.715	2.936	10.264	3.10E-05***	5–9.82 (+) 12.72–21 (–)	6.45–12.16 (>Con) 17.3–21 (<Con)
		LB	2.787	2.964	4.778	2.63E-03**	8.86–15.05 (+) 18.27–21 (–)	
	Females	Con	1.993	2.364	2.834	0.0543		
		MS	2.051	2.423	3.754	0.0197*	14.89–21 (–)	
		LB	2.92	2.995	9.74	5.58E-06***	8.78–15.21 (+) 16.34–21 (–)	6.21–10.39 (<Con) 13.36–19.31 (>Con)
Average peak frequency (kHz)	Males	Con	1.983	2.358	13.79	0.00000138***	5–15.37 (+)	
		MS	2.916	2.994	21.43	1.66E-11***	8.54–16.26 (+) 18.99–21 (–)	6.61–11.27 (<Con) 14.49–19.47 (>Con)
		LB	1	1	12.33	0.000645***	5–21 (+)	12.24–16.58 (<Con)
	Females	Con	1.598	1.937	5.525	0.00385**	5–14.89 (+)	
		MS	2.395	2.736	5.693	1.12E-03**	6.53–13.04 (+)	
		LB	2.59	2.872	4.147	0.02148*	5–9.9 (+)	8.46–11.43 (>Con)
Average bandwidth (kHz)	Males	Con	2.406	2.744	6.609	0.002122**	5–10.47 (+) 15.21–17.54 (–)	
		MS	2.479	2.798	7.678	7.41E-04***	5–10.07 (+) 14.17–21 (–)	
		LB	2.256	2.62	8.652	0.000104***	5–11.75 (+) 16.18–21 (–)	
	Females	Con	2.171	2.539	5.909	0.00193**	5–11.19 (+) 16.18–21 (–)	
		MS	2.732	2.944	6.2	2.44E-03**	5–9.82 (+) 13.36–17.14 (–)	
		LB	2.831	2.977	11.848	4.63E-07***	7.17–14.57 (+) 16.1–21 (–)	6.21–8.38 (<Con) 13.36–18.91 (>Con)

*Effective degrees of freedom (edf) indicates the degree of non-linearity of the age-outcome relationship (edf = 1 for linear relationships). Periods of age-related change based on derivates of GAM models are denoted by (+) for increases and (–) for decreases. Significant differences between MS or LB and Con are based on differences in GAM predictions and 95% confidence intervals. *p < 0.05, **p < 0.01, ***p < 0.001. n = 9–10.*

(SST: Con: $F = 2.834$, $p = 0.0543$; MS: $F = 3.754$, $p = 0.0197$*; LB: $F = 9.74$, $p < 0.001$***; **Figure 3B**). The model predicts a decrease in USVs in MS females from P14.89-21. LB USVs increased between P8.78 and P15.21, and subsequently decreased between P16.34 and P21. The model predicts that LB emitted fewer calls than Con between P6.21 and P10.39, and more calls than Con between P13.36 and P19.31.

Average Bandwidth and Average Peak Frequency

An average bandwidth (maximum frequency – minimum frequency) and average peak frequency across all calls for a given subject were calculated. In males, USVs in all rearing groups increased in average peak frequency as age increased (SST: Con: $F = 7.396$, $p = 0.001$**; MS: $F = 7.521$, $p < 0.001$***; LB: $F = 6.564$, $p < 0.001$***, **Figure 4A**). Specifically, age-related increases occurred between P5 and P15.37 in Con, between P8 and P16.26 in MS, and between P5 and P21 in LB. Interestingly, the GAM was reduced to a linear relationship in LB males, as indicated by an effective degrees of freedom (edf) equal to 1. MS peak frequency was lower than Con between P6.61 and P11.27, and greater than Con between P14.49 and P19.47. LB peak frequency was less than Con from P12.24-16.58. Males in all rearing conditions also increased the average bandwidth of calls across development, and no group was significantly different from Con at any age (SST: Con: $F = 6.609$, $p = 0.002$**; MS: $F = 7.678$, $p < 0.001$***; LB: $F = 6.8.652$, $p < 0.001$***, **Figure 4C**). Con males increased USV bandwidth from P5-P10.47 and decreased from P15.21-P17.54. Similarly, bandwidth increased in MS males between P5 and P10.07 and decreased from P14.17 until P21. LB USVs increased in bandwidth from P5-P11.75 and decreased from P16.18 until P21.

In females, there was a developmental increase in the average peak frequency of USVs in all groups (SST: Con: $F = 3.889$, $p = 0.022$*; MS: $F = 4.13$, $p = 0.023$*; LB: $F = 4.89$, $p = 0.006$**, **Figure 4B**). This increase occurred from P5 to P14.89 in Con, P6.53 to P13.04 in MS, and P5 to P9.9 in LB-reared animals, and LB peak frequency was greater than Con between P8.46 and P11.43. The average bandwidth of female calls also depended on age in all rearing conditions (SST: Con: $F = 5.909$, $p = 0.014$*; MS: $F = 6.2$, $p = 0.002$*; LB: $F = 11.848$, $p < 0.001$***, **Figure 4D**), with Con pups increasing between P5 and P11.19 and decreasing from P16.18 to P21. The model predicted an increase in MS bandwidth between P5 and P9.82 and decrease between P13.37 and P17.14. In LB females, bandwidth increased from P7.17 until

TABLE 3 | Summary statistics of USV call types as a percentage of total calls emitted.

			edf	*F*	*p*	Periods of age-related changes	Age periods significantly different from Con
Complex (%)	Males	Con	1.027	4.136	0.045*	9.66–19.87 (+)	
		MS	1.671	0.934	3.99E-01		5–6.13 (>Con)
		LB	1.663	0.819	0.437		
	Females	Con	1	6.433	0.0125*	5–21 (+)	
		MS	1.469	0.527	6.39E-01		
		LB	1.609	0.969	0.3219		5–8.14 (>Con) 14.73–20.84 (<Con)
Constant (%)	Males	Con	2.654	15.226	0.0000001***	5–10.87 (–)	
		MS	2.115	8.302	2.87E-04***	5–11.43 (–)	
		LB	2.634	16.942	1.97E-08***	5–11.19 (–)	
	Females	Con	2.437	17.266	2.53E-08***	5–11.51 (–)	
		MS	2.199	7.085	0.000928***	5–11.11 (–)	
		LB	2.106	11.647	8.57E-06***	5–12.56 (–)	
Downsweeps (%)	Males	Con	1.025	8.575	0.00351**	5–21 (–)	
		MS	1.996	1.407	1.88E-01**	9.42–10.31 (–)	20.52–21 (>Con)
		LB	2.789	2.535	0.08201		
	Females	Con	1	0.965	0.3281		
		MS	1	0.175	6.76E-01		
		LB	2.797	2.577	0.0722		
Fragmented (%)	Males	Con	2.498	3.516	0.05533		
		MS	2.87	12.032	2.19E-06***	5–9.66 (+) 11.19–18.19 (–)	7.41–11.99 (>Con) 18.59–20.2 (<Con)
		LB	2.205	6.389	8.26E-04***	5–12.16 (+)	5.96–8.3 (<Con) 16.34–19.47 (>Con)
	Females	Con	1.686	0.844	0.451725		
		MS	2.856	6.804	0.000667***	5–9.34 (+) 11.03–16.9 (–)	7.01–12.48 (>Con) 17.46–19.87 (<Con)
		LB	2.878	9.77	5.59E-06***	8.46–15.21 (+) 16.74–21 (–)	5–9.98 (<Con) 12.8–19.95 (>Con)
Inverted-U or Upright-U (%)	Males	Con	1	0.751	0.388		
		MS	1	2.274	1.34E-01		
		LB	1	0.815	0.369		
	Females	Con	1	0.435	0.5111		
		MS	1.743	1.634	1.87E-01		
		LB	2.337	2.814	0.0842		
Upsweeps (%)	Males	Con	2.143	4.489	0.00622**	6.53–12.4 (+)	
		MS	2.747	10.151	5.64E-06***	8.7–16.18 (+)	7.17–9.9 (<Con) 18.27–19.79 (>Con)
		LB	1	7.386	0.00763**	5–21 (+)	12.32–14.73 (<Con)
	Females	Con	1.822	3.032	0.04538*	6.29–10.95 (+)	
		MS	1.576	5.804	3.15E-03**	5.88–15.53 (+)	
		LB	1.135	2.281	0.10153		

*GAMs were performed for males and females separately, comparing MS or LB to Con. Effective degrees of freedom (edf) indicates the degree of non-linearity of the age-outcome relationship (edf = 1 for linear relationships). Periods of age-related change based on derivates of GAM models are denoted by (+) for increases and (–) for decreases. Significant differences between MS or LB and Con are based on differences in GAM predictions and 95% confidence intervals. Bolded call types indicate an effect of both age and rearing. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 9–10.*

P14.57 and decreased from P16.1 until P21. The model predicted that in females, LB bandwidths were, on average, lesser than Con between P6.21 and P8.38, and greater than Con between P13.36 and P18.91.

Additional GAM analyses comparing males and females within rearing conditions demonstrated age-related changes in both sexes in all rearing conditions. In Cons, there were no differences between males and females. In MS, female peak frequency was greater than males between P7.01 and P12.08, but less than males between P18.11 and P20.84. In LB, female peak frequency was greater than males between P8.54 and P13.2.

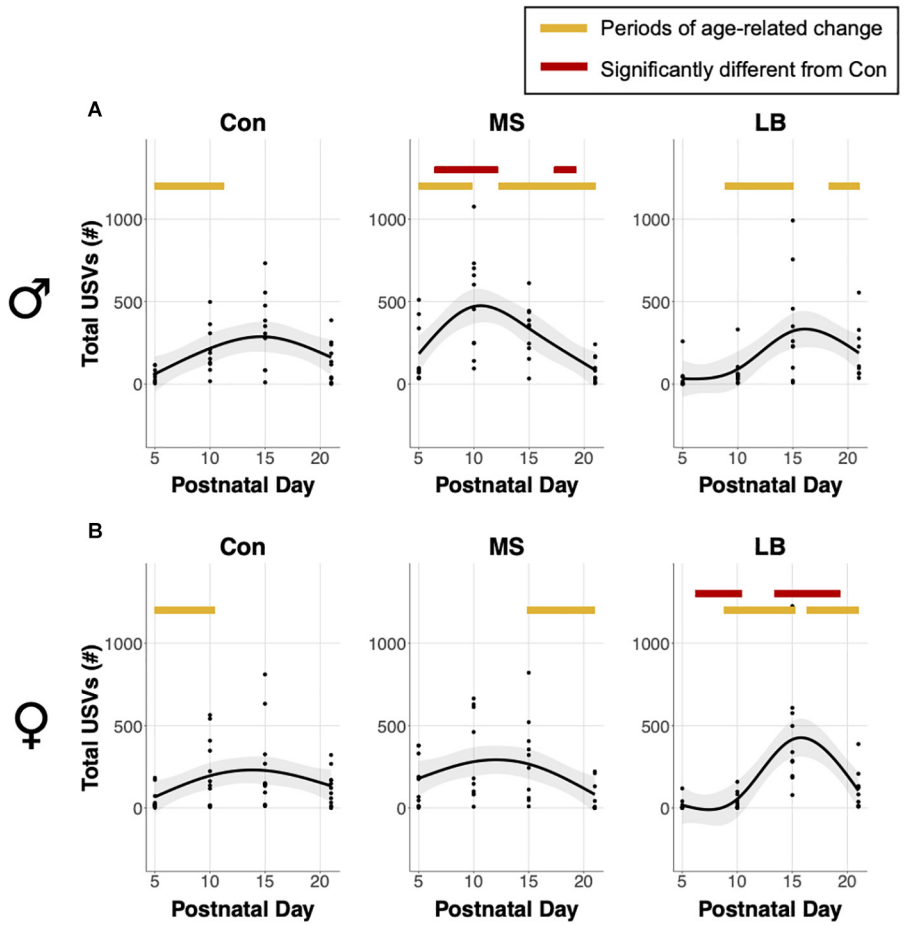

FIGURE 3 | Effects of age and maternal separation (MS) or limited bedding (LB) on total ultrasonic vocalizations (USVs) emitted in **(A)** males and **(B)** females. Shaded areas represent 95% confidence intervals. Orange bars indicate periods of significant age-related change based on the derivative of the GAM model fit. Red bars indicate periods significantly different from control (Con) based on differences between GAM models and confidence intervals. n = 9–10.

Maternal Behavior

Entropy Rate

To assess the overall predictability of maternal behavior, entropy rates were calculated at 0830, 1430, and 2330. Entropy rates were not observed to be affected by MS or LB at any time point [0830: $F_{(2,32)} = 0.02991$, $p = 0.9706$, $\eta_p^2 = 0.002$; 1430: $F_{(2,31)} = 0.5729$, $p = 0.5701$, $\eta_p^2 = 0.0038$; 2330: $F_{(2,28)} = 0.8804$, $p = 0.4266$, $\eta_p^2 = 0.0634$; **Figure 5**]. To ensure that entropy was not attributable to variability in pup weight, ANCOVAs were conducted for maternal entropy at each of three time points with pup weight at P8 as a covariate (**Supplementary Table 3**). Pup weight did not covary with maternal entropy scores ($p > 0.05$ at all time points for both males and females).

On Nest Duration

There was no effect of rearing on the total time spent on the nest at 0830 [$F_{(2,32)} = 1.281$, $p = 0.2926$, $\eta_p^2 = 0.0787$] or 2330 [$F_{(2,27)} = 1.961$, $p = 0.1618$, $\eta_p^2 = 0.1356$] (**Figure 6A**). At 1430, data failed the Shapiro-wilk test of normality ($p = 0.0214$), but a Kruskal-Wallis test showed that rearing affected on-nest duration [$H_{(2)} = 7.397$, $p = 0.0248$, **Figure 6A**]. *Post-hoc* comparisons reveal that this effect is likely driven by increased time spent on the nest by LB dams compared to Con dams ($p = 0.0255$).

Licking and Grooming

There was no observed effect of rearing on the total time spent licking and grooming (LG) at any time point [0830: $F_{(2,31)} = 0.7399$, $p = 0.4860$, $\eta_p^2 = 0.0485$; 1430: $F_{(2,31)} = 1.655$, $p = 0.2087$, $\eta_p^2 = 0.1024$; 2330: $F_{(2,26)} = 0.1864$, $p = 0.8311$, $\eta_p^2 = 0.0153$] (**Figure 6B**), and no effect on the total number of LG bouts [0830: $F_{(2,29)} = 1.275$, $p = 0.1952$, $\eta_p^2 = 0.0835$; 1430: $F_{(2,29)} = 0.5690$, $p = 0.5723$, $\eta_p^2 = 0.0378$; 2330: $F_{(2,27)} = 0.2448$, $p = 0.7847$, $\eta_p^2 = 0.0192$] (**Figure 6B**).

Nursing

Arched-back nursing (ABN) and passive nursing bouts were quantified separately. There was a significant effect of rearing on the total time dams spent passively nursing at 1430 [$F_{(2,31)} = 4.105$, $p = 0.0269$, $\eta_p^2 = 0.0629$], and *post-hoc* comparisons revealed that this effect was driven by an increase in passive nursing in LB dams compared to MS dams ($p = 0.0212$)

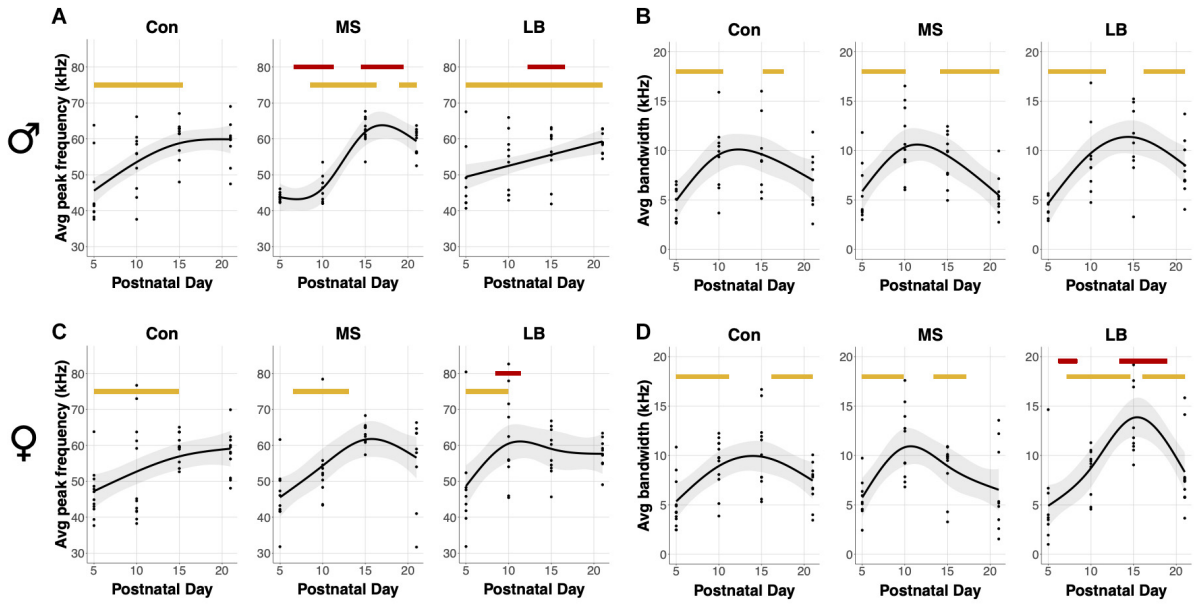

FIGURE 4 | Effects of age and maternal separation (MS) or limited bedding (LB) on peak frequency and bandwidth averaged across all USVs for each subject. Age related increases or decreases are indicated by orange bars. **(A)** In males, peak frequency was decreased by MS P6.61-P11.27 and increased by MS P14.49 and P19.47. LB peak frequency was decreased in males P12.24-16.58. **(B)** There was no effect of MS or LB on developmental changes in bandwidth in males. **(C)** In females, LB increased peak frequency from P8.46-P11.43. **(D)** LB decreased the bandwidth of female calls from P6.21-P8.38 and increased the bandwidth from P13.36-P18.91 n = 8–10.

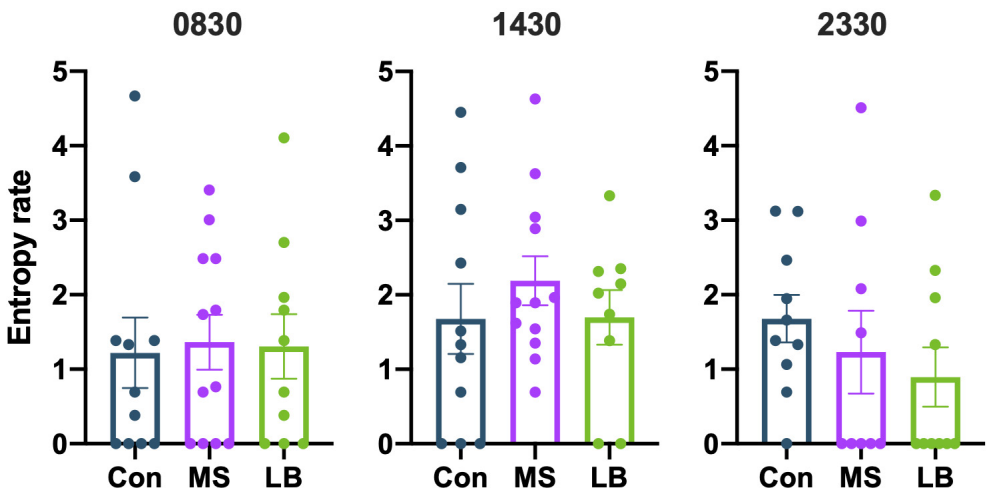

FIGURE 5 | Maternal separation (MS) and limited bedding (LB) did not affect the entropy of maternal behavior sequences at 0830, 1430, or 2330. n = 9–12.

(**Figure 7A**). There were no effects of rearing on the time dams spent passive nursing at 0830 [$F_{(2,31)}$ = 2.896, p = 0.0713, η_p^2 = 0.1665] or 2330 [$F_{(2,27)}$ = 0.9960, p = 0.3835, η_p^2 = 0.0738], and no effect on the number of passive nursing bouts [0830: $F_{(2,31)}$ = 0.6294, p = 0.54, η_p^2 = 0.0416; 1430: $F_{(2,31)}$ = 0.3072, p = 0.7379, η_p^2 = 0.0207; 2330: $F_{(2,27)}$ = 0.2343, p = 0.7928, η_p^2 = 0.0184] (**Figure 7B**).

The total time spent ABN was not affected by rearing condition at any time point [0830: $F_{(2,31)}$ = 0.4701, p = 0.6296, η_p^2 = 0.0314; 1430: $F_{(2,31)}$ = 0.5839, p = 0.5641, η_p^2 = 0.0387; 2330: $F_{(2,27)}$ = 2.210, p = 0.1306, η_p^2 = 0.1503] (**Figure 7C**). However, rearing affected the number of ABN bouts at 1430 [$F_{(2,31)}$ = 3.904, p = 0.0315, η_p^2 = 0.2121], but not at 0830 [$F_{(2,31)}$ = 0.9739, p = 0.3896, η_p^2 = 0.0629] or 2330 [$F_{(2,27)}$ = 2.090, p = 0.1447, η_p^2 = 0.1433] (**Figure 7D**). This effect was driven by an increase in ABN bouts in MS dams compared to Con (p = 0.0241) (**Figure 7D**).

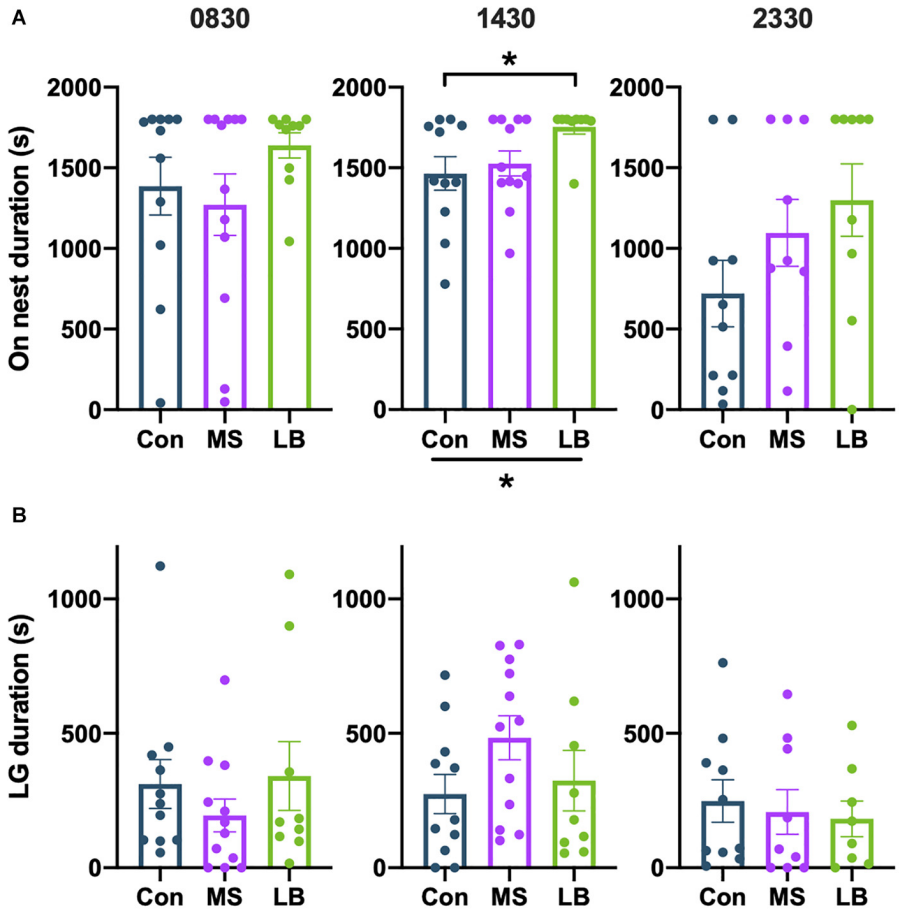

FIGURE 6 | Effects of rearing on **(A)** time spent on the nest and **(B)** time spent licking and grooming (LG). LG was increased in LB dams compared to Con at 1430. *$p < 0.05$. $n = 9$–12.

Maternal Entropy Predicted Total USVs Emitted by Females at P21

ANCOVA were conducted to determine whether total USVs at weaning were related to the predictability of postnatal maternal care. Statistics for all ANCOVA can be found in **Supplementary Table 4**. In females, entropy rate at 1430 was negatively correlated with the number of USVs emitted by pups at P21 ($p = 0.012$; $\eta^2 = 0.1973$) (**Figure 8B**), and there was no effect of rearing on total USVs after correcting for entropy rate. In males, there was no effect of entropy rate at any time on the total number of USVs emitted ($p = 0.316$; $\eta^2 = 0.0739$) (**Figure 8A**).

DISCUSSION

Identifying specific factors of adverse experiences that contribute to later-life consequences will help to generalize findings from rodent studies for use in the human realm. To this end, the current study determined the effects of two adversity models, MS and LB, on USV emissions and patterns of maternal care. We aimed to characterize typical developmental trajectories of infant communication, and the extent to which maternal behavior and adversity impacted USV maturation. Overall, our findings show that MS and LB altered total USV emissions, peak frequency, and four call types in a sex-specific manner, and total USVs at P21 were associated with the predictability of postnatal maternal care. This final finding highlights the impact of a specific aspect of maternal behavior on behavioral development in all rearing conditions.

There are limitations to the study design that should be noted when interpreting the findings. First, pups were recorded in small cups in a water bath at P5 and P10 and in small cages at P15 and P21. Recording in the water bath at P5 and P10 was necessary since reduced core temperature has an effect on USVs that is distinct from the effects of isolation (Hahn and Lavooy, 2005), and this study aimed to test the USV response due to the experience of isolation alone, and not additional environmental stressors. To maintain consistency between the two contexts, home cage bedding was provided in both to limit any novel olfactory cues. Therefore, although the recording protocol attempted to minimize changes between conditions at P5/P10 and P15/P21 while maintaining core body temperature at all times recorded, age-related changes in USV trajectory should be interpreted with this consideration. Another limitation is the

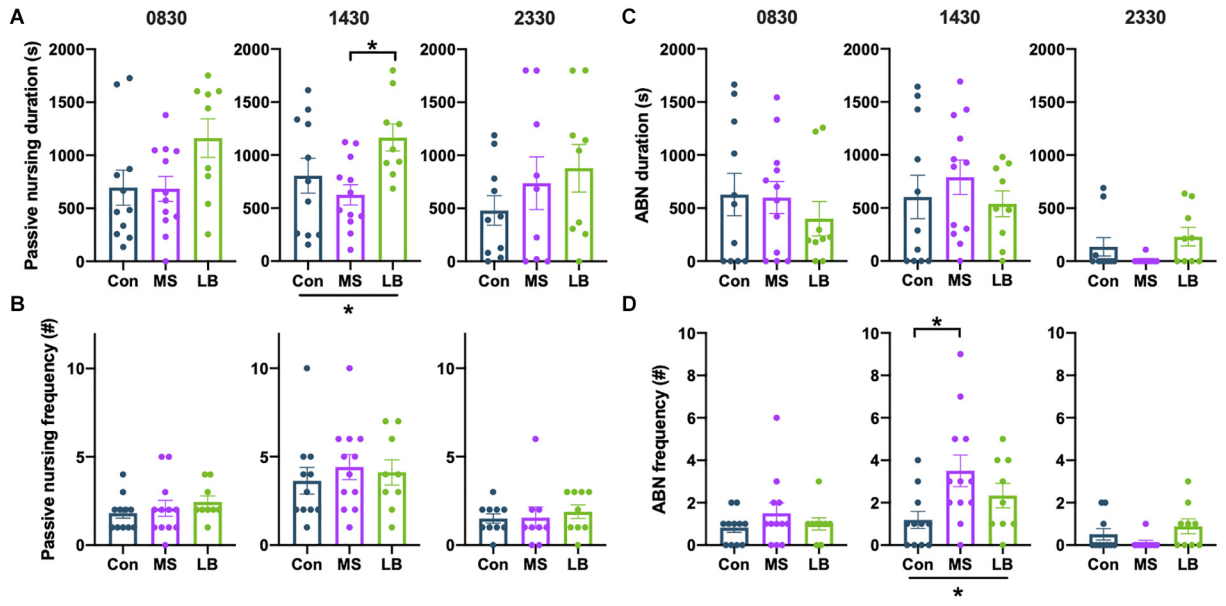

FIGURE 7 | Effects of maternal separation (MS) or limited bedding (LB) on passive nursing and arched-back nursing (ABN) durations **(A,C)** and frequencies **(B,D)** at 0830, 1430, and 2330. At 1430, LB dams spend more time ABN than MS dams, but nursing did not differ at 0830 or 2330. At 1430, MS dams were more frequently initiating ABN bouts than Con dams, but the total duration of ABN did not differ. *$p < 0.05$. $n = 9$–12.

difference in the timelines of MS and LB paradigms. The P2-P20 MS timeline was implemented to align with the methods used in previous findings from our lab, while the P2-P14 is more typical for the LB protocol (Walker et al., 2017). Since LB ended on P14 and pup USVs were recorded on P15, changes in USVs seen during this time may be a response to returning to standard rearing cages. For these reasons, we avoided directly comparing the USV development curves of MS and LB, and instead interpret the results in relation to the control group. For maternal behavior, however, both adversity groups had undergone MS or LB for the same amount of time at the time of recording (P8-P9), so direct comparisons are made between Con, MS, and LB. Future work will determine the extent to which MS and LB affect USV trajectories if their timelines are identical.

We report an age-dependent increase in USV emissions during the first half (through P12) of the preweaning period in males and females reared in standard conditions. Heightened USV emissions at P11-P12 temporally aligns with the approximate end of the stress hyporesponsive period (SHRP) in rodents. In rats, the SHRP is the estimated period between P4 and P14 characterized by low basal corticosterone levels and a suppressed neuroendocrine response to stressors (Sapolsky and Meaney, 1986). Our findings align with others who have shown that vocalizations are highest between P6 and P12 (Noirot, 1968; Insel et al., 1986; Brudzynski, 2019). Increases in USVs during this period with maximum emission rates emerging by P15 could therefore indicate maturation of the HPA axis.

In males undergoing MS, USV emissions were increased earlier in the SHRP compared to Con, consistent with a previous report that male pups undergoing MS emitted more USVs than controls at P12 (Kaidbey et al., 2019). Prolonged separation periods from the dam during the SHRP invokes corticosterone release in otherwise quiescent pups (Levine, 2001); therefore enhanced vocalization rates at a younger age while undergoing MS may point to a change in typical development of HPA axis responsivity (Branchi et al., 2001; Hofer et al., 2001; Levine, 2001; Dirks et al., 2002). Notably, we found this response to MS to be specific to males. No effect of MS was seen in females, while USVs were initially suppressed by LB until P10. After approximately P14, LB females emitted more USVs than Con. The initial suppression of USVs in females may be due to sex differences in typical HPA axis maturation (Yoshimura et al., 2003) and the curvilinear fashion by which corticotropin releasing factor (CRF) modulates USVs; CRF at moderate levels dose-dependently invoke USVs, but USVs are eventually quieted with high levels of CRF (Dirks et al., 2002). Sex differences in maternal care directed at individual pups may also contribute to differences in USVs (Richmond and Sachs, 1983). Although not measured here, females have been shown to receive higher levels of maternal behaviors resembling abuse when reared in adversity models, while males receive more tactile stimulation and general contact (Keller et al., 2019). Decreased USV emissions in LB females may represent an adaptive avoidance strategy if maternal contact is abusive or painful (Moriceau et al., 2009); however, deconstructing the specific factors of LB that lead to female-specific effects will require analysis of maternal care on individual pups.

Peak frequency, bandwidth, and sonographic structure of USVs were also altered depending on sex and adversity type. There was a developmental increase in average peak frequency beginning at P5 and continuing until approximately P15. In

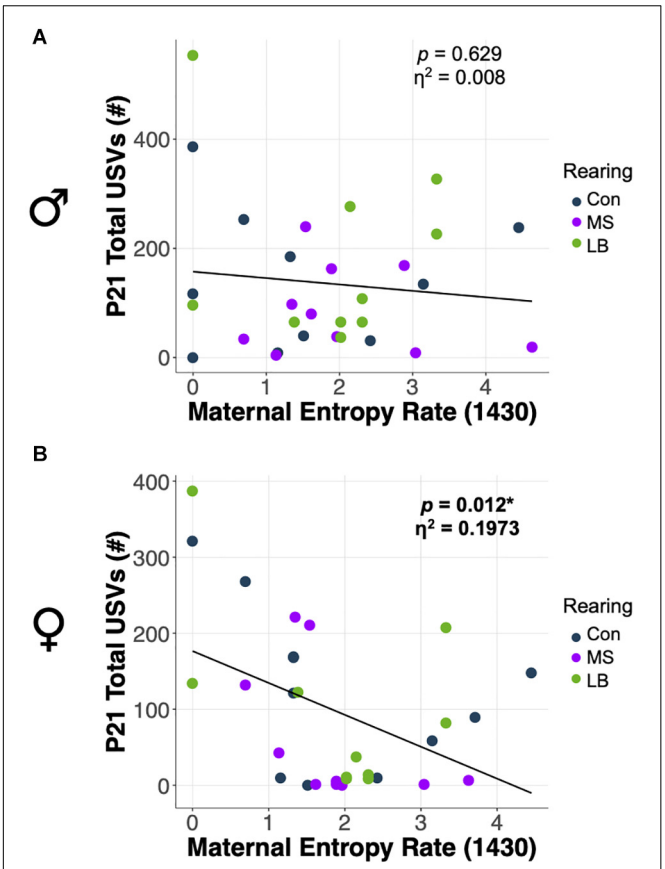

FIGURE 8 | Effects of maternal entropy rate at 1430 on total USV emissions at P21 in **(A)** males and **(B)** females. *$p < 0.05$, $n = 10–11$.

males, MS initially hindered this increase, but later, between P15 and P20, peak frequency was elevated compared to Con. Comparing males to females within each rearing condition showed that male peak frequency was higher than females during this period. Kaidbey et al. (2019) found a similar pattern of higher USV frequency in MS pups during both an initial and secondary separation from the dam. In females, we found that peak frequency was elevated by LB, but not MS, and this elevation occurred around P10. Again, this demonstrates an effect of MS in males and LB in females.

Since the frequency of a vocalization is influenced by its sonographic structure (Ehret, 2005), we also quantified USVs by proportions of call types. Identifying developmental changes in particular call types could reveal acoustic characteristics that are functionally relevant at particular ages (Brudzynski et al., 1999). We found that the proportion of specific call types emitted by Con males and females fluctuated with age. Brudzynski et al. (1999) argues that developmental profiles of specific USV waveforms in the rat could indicate adaptive significance for communication and facilitation of maternal care. The occurrence of complex calls, characterized by recurrent frequency modulation, increased over development and composed more than 50% of all call types by P21. Complex calls could thereby signify a more mature vocal repertoire.

Reported effects of ELA on maternal behavior have been mixed and seem to largely depend on the type of adversity used, measures assessed, and particular experimental procedures which widely vary between labs (Orso et al., 2019). Recently, more attention has been given to signs of fragmented or erratic maternal behavior, especially in LB paradigms, which are argued to induce unpredictable care patterns (Molet et al., 2016). We did not observe effects of MS or LB on the predictability of maternal behavioral sequences as measured by entropy rates, yet it should be noted that others have recorded maternal behavior over 60 or 180-min periods, while we only observed 30-min windows. Further, we did not code off-nest behaviors—aside from self-grooming. Including more diverse behaviors in the analysis and extending the observation period could produce results more sensitive to potential behavioral changes and should be considered for future studies. Although we did not find group differences in entropy rate, we hypothesized that entropy rate variability is linked to pup USV emissions. In fact, we show that entropy at 1430 negatively correlated with total USVs in females at P21. This suggests that maternal entropy rate could be a common factor of the rearing environment, regardless of adversity condition, that influences USVs at weaning age. To test the validity of this correlation between USVs and maternal care, experiments directly testing the maternal response to USVs must be performed. An important addition to this line of work will be determining whether differences in USVs due to rearing condition or individual differences impact maternal retrieval behavior.

Previous investigations in both humans and rodents have found certain features of distress vocalizations to be functionally significant in parent-infant relationships (Blumberg and Sokoloff, 2001; Zeskind et al., 2011). In humans, spectral features of infant cries are associated with developmental disturbances and have been used as an early identifier of medical conditions and developmental disorders (LaGasse et al., 2005; Zeskind et al., 2011; Sheinkopf et al., 2012). For example, shifts to higher fundamental frequencies in infant crying is associated with prenatal cocaine exposure (Zeskind et al., 2011) and are more pronounced in infants later diagnosed with autism spectrum disorder (Sheinkopf et al., 2012). Additionally, infants born prematurely are at an increased risk for vocal and cognitive impairments characteristic of developmental disorders, which may be linked to prolonged periods of maternal separation in the neonatal intensive care unit (Beebe et al., 2018). Family Nurture Intervention programs curtail these deficits by increasing quality mother-infant interactions through scent exchange, direct touch, and vocal communication from mother to infant (Welch et al., 2015). While rodent vocalizations are not analogous to human cries, comparing similar features of species-specific distress signals can validate these models for translational approaches to studying interventions for neurodevelopmental disorders. Indeed, the ability

for preclinical research to implement characterizations of socially relevant communication (i.e., USVs) may provide critical translational insight into the developmental dynamics of communication, which are often found to be disrupted in developmental and psychiatric disorders.

Although rodent models of ELA are widely used, discussions of potential differences in their effects on the offspring experience are often ignored. Overall, this study demonstrates that infant vocalizations in rats follow a developmental pattern that can be referenced to decipher impacts of the early environment on maturation. Two different adversity paradigms each affected maternal behavior and pup communication strategies, and predictable maternal behavior was a common factor influencing female pup development. Assessing ultrasonic communication in rodents can serve as a translational tool for researchers to better characterize early neurobehavioral disturbances, contributing to our understanding of the etiology of psychiatric vulnerabilities in humans exposed to ELA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Northeastern Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LG, JH, and HB conceptualized and designed the experiments. LG, AV, and JLH collected data. LG performed statistical analysis and wrote the original draft. AV, JLH, JH, and HB edited the original draft. All authors contributed to the article 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/fnhum.2021.632702/full#supplementary-material

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Infantile Iron Deficiency Affects Brain Development in Monkeys Even After Treatment of Anemia

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A high percent of oxidative energy metabolism is needed to support brain growth during infancy. Unhealthy diets and limited nutrition, as well as other environmental insults, can compromise these essential developmental processes. In particular, iron deficiency anemia (IDA) has been found to undermine both normal brain growth and neurobehavioral development. Even moderate ID may affect neural maturation because when iron is limited, it is prioritized first to red blood cells over the brain. A primate model was used to investigate the neural effects of a transient ID and if deficits would persist after iron treatment. The large size and postnatal growth of the monkey brain makes the findings relevant to the metabolic and iron needs of human infants, and initiating treatment upon diagnosis of anemia reflects clinical practice. Specifically, this analysis determined whether brain maturation would still be compromised at 1 year of age if an anemic infant was treated promptly once diagnosed. The hematology and iron status of 41 infant rhesus monkeys was screened at 2-month intervals. Fifteen became ID; 12 met clinical criteria for anemia and were administered iron dextran and B vitamins for 1–2 months. MRI scans were acquired at 1 year. The volumetric and diffusion tensor imaging (DTI) measures from the ID infants were compared with monkeys who remained continuously iron sufficient (IS). A prior history of ID was associated with smaller total brain volumes, driven primarily by significantly less total gray matter (GM) and smaller GM volumes in several cortical regions. At the macrostructual level, the effect on white matter volumes (WM) was not as overt. However, DTI analyses of WM microstructure indicated two later-maturating anterior tracts were negatively affected. The findings reaffirm the importance of iron for normal brain development. Given that brain differences were still evident even after iron treatment and following recovery of iron-dependent hematological indices, the results highlight the importance of early detection and preemptive supplementation to limit the neural consequences of ID.

Keywords: anemia, infancy, brain development, neuroimaging, diffusion tensor imaging, gray matter, monkey

INTRODUCTION

Sadly, the optimal development of many children worldwide is still compromised by an inability to obtain sufficient food and to consume a nutritionally complete diet (Petry et al., 2016; Armitage and Moretti, 2019). Beyond the known effects on growth and general health, poor nutrition can worsen the impact of infection and poverty (Abioye et al., 2019). In addition to the importance of macronutrient intake, extensive research has demonstrated a need for specific micronutrients. Iron has been a particular focus of attention because of the high prevalence of ID in pregnant women and young children, and its essential role in energy metabolism (Gupta et al., 2017). Numerous studies have shown that both too little and too much iron can affect cellular functions throughout the body, especially during the vulnerable fetal and infancy periods when developmental needs for iron are high due to rapid growth (Kwik-Uribe et al., 2000; Yu et al., 2011). The potential for ID to affect neural processes is a major pediatric concern because approximately 60% of oxidative energy in growing infants is devoted to brain development (Steiner, 2019). Iron is essential for oxygen transport and delivery, is involved in mitochondrial energy production through its role in electron transport/oxidative phosphorylation, and it is a critical co-factor and catalyst in many synthetic pathways (Bastian et al., 2019).

To ensure an adequate postnatal supply of iron, many mammalian species, including humans, rely on a 2-stage sequence. First, substantial amounts of maternal iron are transferred across the placenta before birth, which is then supplemented postnatally by the enteral iron in breast milk and later by the iron in solid foods after weaning (Georgieff, 2020). During late pregnancy, placental receptors actively bind iron in maternal blood, and thus can partially protect the fetus from maternal anemia, but the mother's diet and health can still affect the iron present in hemoglobin and ferritin reserves in tissue at term (Lubach and Coe, 2006). Maternal anemia, placental insufficiency, gestational diabetes, and premature birth have all be found to affect the iron level in neonates (O'Brien et al., 2003; Dosch et al., 2016; Georgieff, 2020). In addition, maternal stress during pregnancy can increase the risk for infantile ID in both animals and humans (Coe et al., 2007; Rendina et al., 2018). These findings have contributed to a paradigm shift in prenatal care. Iron supplements are now routinely recommended both for the mother and to ensure sufficient iron transfer to prevent postnatal ID in infants (Beard et al., 2007; Georgieff, 2020).

Manipulating the dietary supply of iron during pregnancy was the approach used to create a naturalistic animal model of infantile ID. Female monkeys were provided with a level of iron that met the recommended nutrient requirements for non-human primates (National Research Council [NRC], 2003), but is not sufficient to entirely fulfill the higher requirements of the gravid female. It simulates a gestation without the recommended iron supplementation, which is common in low-income countries as well as among lower SES families in high-resource countries, who may not have ready access to prenatal care until late gestation (Armitage and Moretti, 2019). We have shown previously that this prenatal diet results in 20–30% of infant monkeys developing a growth-related ID after birth during the nursing phase, before they recover gradually by consuming the iron in solid foods (Coe et al., 2013). This prevalence is comparable to the rates of anemia caused by iron deficiency in many lower income countries (Petry et al., 2016). Because the infantile anemia in this monkey model is due exclusively to diet, and emerges as the infant's postnatal growth needs exceed reserves, it has been possible to demonstrate specific effects of ID on peripheral physiology as well as an iron-related reduction in energy metabolism in the CNS (Geguchadze et al., 2008; Patton et al., 2012; Sandri et al., 2020). There is also decreased dopamine activity and upregulated norepinephrine release in the CNS of infant monkeys who had been anemic (Coe et al., 2009), which is similar to the decreased brain dopamine induced in rodent models (Pino et al., 2017). The current analysis addressed the additional question of whether treating an anemic infant with iron at the time of diagnosis could prevent sustained effects on the brain when measured later.

Many prior assessments of human infants and children have documented effects of ID on neurodevelopment (Lozoff et al., 2000, 2006; Georgieff, 2017). The deficits in attentional and emotional processes, sensory processing and neuromotor performance suggest the neural effects are widespread (McCann et al., 2020). However, definitively proving that the long-lasting effects are due solely to iron can be challenging in humans because there are often other dietary deficiencies as well as confounding social factors. One functional MRI evaluation of young adults diagnosed earlier to be anemic during infancy indicated there may be long-lasting effects on neural connectivity (Algarin et al., 2017). That conclusion would concur with experimental models demonstrating that ID during early infancy can affect neurogenesis, synaptogenesis, and dendritogenesis in rodent pups and piglets (Wu et al., 2008; Ferreira et al., 2019).

Iron is especially needed for myelin synthesis by oligodendrocytes, leading to the conclusion that axonal myelination may be a sensitive sentinel indicator of the compromised neurodevelopment (Connor and Menzies, 1996; Todorich et al., 2009). For that reason, our neuroimaging analysis included diffusion tensor imaging (DTI), which can detect deficits in microstructural integrity and myelin maturation. Our *a priori* hypothesis was that later-maturing anterior tracts would be more impacted because the growth-related ID in monkeys typically emerges relatively late between 4 and 8 months of age. Experimental models of early ID in young piglets indicated there were additional effects on GM in limbic areas and throughout the cortex (Leyshon et al., 2016). Therefore, structural MRI analyses were used to determine whether the transient anemia that occurs in older infant monkeys at the end of their nursing period would also affect total brain volume and regional GM volumes, even if treated quickly by administering iron.

MATERIALS AND METHODS

Subjects

Forty-one infant rhesus monkeys (*Macaca mulatta*) born to multiparous adult females were evaluated in this study (19 female and 22 male). These monkeys were part of an established

breeding colony imported from India over 50 years ago, but then bred at this facility for over 10 generations (Beck et al., 2019). All were from full-term, unassisted vaginal deliveries and nursed by their mothers through 6 months of age. Between 6 and 7 months of age, the older weanlings were rehoused into small social groups with other juveniles until 12 months of age when the neuroimaging scans were acquired. The scan of one anemic monkey was excluded because of poor quality, resulting in a total of 40 for the final analyses of volumetric and DTI data. These husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Housing

During the nursing phase, the infants were raised by their mothers in standardized cages and then moved subsequently to identical double cages with 4 juvenile peers, in order to ensure that housing conditions were similar for all animals across the first year of life. Adult females and older juveniles were fed the same diet (5LFD, LabDiet, St. Louis, MO, United States), supplemented three times per week with fruit and vegetables. Filtered tap water was available *ad libitum*, and tested to verify it was lead-free. The ingredient composition of this diet has been published previously, but important details are provided in **Supplementary Table 1**. The mean iron concentration in the biscuits (225 mg/kg) meets published nutrient requirements for adult monkeys (National Research Council [NRC], 2003), but does not ensure that all pregnant females will transfer equivalently high levels of iron to their infants (Lubach and Coe, 2006; Coe et al., 2013). The photoperiod was set at 16 h light/8 h dark, with lights turned on at 0600 year-round so that all blood samples would be obtained at the same point in the diurnal cycle.

Infant Assessments

Blood samples (<3 mL) were collected at 2-month intervals across the first year of life in order to evaluate the infants' hematology and several iron-related indices. Specimens were collected between 0930 and 1100 via femoral venipuncture. For the purposes of this analysis, only the mean corpuscular volume (MCV) and hemoglobin (Hgb) levels are reported, but we have shown previously that both correlate highly with decreases in serum iron, transferrin saturation, the iron-regulator hepcidin, and ferritin levels when an infant is anemic (Coe et al., 2013). There is also a marked increase in zinc protoporphyrin/heme, which reflects a compensatory response of red blood cells (RBC) to low iron. On the same days as the serial blood collection, the infant was weighed and anthropometric measures recorded to assess growth (e.g., head circumference and crown-rump length).

Iron Treatment

If the ID reached clinical criteria for anemia–MCV below 50 fL and a Hgb below 10 g/dL–the young monkey was administered iron dextran (10 mg, IM) and B vitamins (B1, 50 mg, B2, 2.5 mg, Niacinamide, 50 mg, B6, 5 mg, d-Panthenol, 5 mg, B12, 50 mg, IM) weekly until its hematology returned back to the normal range (MCV > 60 fL; Hgb > 10 g/dL). Twelve of the 15 ID infants received treatment. The mean number of IM injections was 6 (range 4–8 weeks until effect). MCV and Hgb screening was determined as part of a Complete Blood Count panel by a CLIA-certified clinical laboratory (UnityPoint Health Meriter Labs, Madison, WI, United States). By verifying the continuation of normal leukocyte values, we also ensured that any decrease in iron was not due to acute infection or illness.

Neuroimaging

MRI scans were acquired when the monkeys were 12–14 months of age (mean age = 374+/−14 days). Briefly, the monkeys received a preanesthetic dose of ketamine (10–15 mg/kg IM) for transport to the imaging facility, followed by dexdomitor (0.015 mg/kg IM) to prevent movement during the scan. The plane of anesthesia was monitored with a pulse oximeter, and body temperature maintained during the 1 h scan. Scans were obtained with a GE MR750 3.0T scanner (General Electric Medical, Milwaukee, WI, United States) using a human 8-channel brain array coil at the Waisman Laboratory for Brain Imaging and Behavior (UW-Madison).

T1 weighted images (124 coronal slices) were acquired using an axial Inversion Recovery (IR) prepared fast gradient echo (fGRE) 3D acquisition sequence (GE BRAVO). The parameters were TI = 450 ms, TR = 8.704 ms, TE = 3.664 ms, flip angle = 12°, matrix = 256 × 256, slice thickness = 0.8 mm, and image gap = −0.4 mm, bandwidth = 31.25 kHz, 80 percent field-of-view in phase encoding direction, 2 averages, with a native voxel resolution 0.55 × 0.55 × 0.8 mm. T2 weighted images (156 sagittal slices) were acquired using a sagittal 3D CUBE FSE sequence with TR = 2500 ms, TE = 81.9 ms, flip angle = 90°, matrix = 256 × 256, 90 percent field of view in the phase encoding direction, slice thickness = 0.6 mm, gap = 0 mm, bandwidth = 62.5 kHz, ARC parallel imaging with a factor of 2 acceleration in both phase encoding and slice encoding directions, voxel size 0.6 × 0.6 × 0.6 mm isotropic resolution.

The DTI protocol used the following parameters: TR = 8000 ms, TE = 83.4 ms, FOV = 16.7 mm, matrix = 128 × 128, 2.6 mm slice thickness with 1.3 mm slice overlap (resolution 1.3 mm × 1.3 mm × 2.6 mm), upsampled to a voxel dimension on the scanner of 0.65 mm × 0.65 mm × 1.3 mm for preprocessing and to an isotropic voxel for diffusion model reconstruction at 0.65 × 0.65 × 0.65 mm. ASSET acceleration factor = 2 in the coronal plane orientation. There were 120 unique gradient directions acquired with 10 baseline images at b = 0 s/mm^2, 10 b = 300 s/mm^2, 40 b = 1000 s/mm^2, and 70 b = 2000 s/mm^2.

Structural Image Processing

T2-weighted images were registered to the T1-weighted images aligned into a common space. After that step, T1w and T2w images were bias-field corrected, and brain masks for skull stripping were created using AutoSeg_3.3.2 (Wang et al., 2014). Following this preprocessing, T1 and T2-weighted images were jointly segmented into GM, WM and cerebrospinal fluid (CSF) using NeosegPipeline_v1.0.8 (Cherel et al., 2015). Subject-specific probabilistic tissue maps were generated from structural multi-atlas templates via deformable registration and then used in an expectation-maximiation tissue segmentation. Lobar parcellation of the tissue segmentation into 24 bilateral lobar brain regions

and eight subcortical GM structures was performed using multi-atlas fusion in AutoSeg_3.3.2 (Wang et al., 2014). All segmentation and parcellation results were quality-controlled visually and no major issues were detected. After the brain parcellation, GM and WM volumes were extracted for each lobar region, and analyzed in addition to total brain volume and GM and WM volumes.

Diffusion MRI Processing

Preprocessing of DWI images included susceptibility-related distortion correction, eddy current and motion correction using topup (Andersson et al., 2003) and eddy_openmp tools (Andersson and Sotiropoulos, 2016). Automatic removal of artifact-rich images was done using DTIPrep (Oguz et al., 2014). A weighted least-square estimation was employed to produce a diffusion tensor image for each subject. Gradients with high b-values (b = 2,000 s/mm^2) were not used in tensor model reconstruction, because higher b-value images (i.e., 2,000 s/mm^2) have a lower signal-to-noise ratio than lower b-values (i.e., 1,000 s/mm^2) and this difference cannot be controlled in the tensor model estimation. Diffusion tensor images were estimated for each monkey using weighted least-square measures. In order to enhance robustness, we chose to exclude gradients from the highest b-value shell (b = 2,000 s/mm^2) for the tensor model estimation. Higher b-value images (b = 2,000 s/mm^2) have lower signal-to-noise ratios than low b-value images (b = 300 and 1,000 s/mm^2) as well as more diffusion artifacts.

Manual brain masks were created from the averaged baseline images of five randomly selected scans (b = 0 s/mm^2) and served as multi-atlas templates to create masks for the other subjects using deformable registration (Avants et al., 2014) and majority voting. DTI images were skull stripped and a study-specific atlas built using DTI Atlas Builder (Verde et al., 2014). Fiber tracts created from a previously created DTI atlas from a different cohort of monkeys[1] were propagated into the study-specific atlas space. These propagated tracts were voxelized and used as seed maps for fiber tractography. Tractography was performed automatically using AutoTract (Prieto et al., 2016), followed by a manual inspection stage to further clean the tract definitions. The process tracts were then mapped back into each subject's DTI image space using deformation fields calculated during the atlast building and parameterized along their arc length to yield four diffusion properties: fractional anisotropy (FA), axial diffusivity (AD), mean diffusivity (MD), and radial diffusivity (RD) using DTI Atlas Fiber Analyzer (Verde et al., 2014). These processes steps yielded diffusion profiles for each tract of interest.

Tracts of Interest

A prior cross-sectional analysis of monkey brain development had demonstrated that larger maturational changes in WM occurred in the temporal and occipital lobes, cingulum and corpus callosum between 1 and 5 years of age (Shi et al., 2013). Thus, for the current analysis, 10 tracts of interest were selected in corresponding brain regions: cingulum (CG), uncinate fasciculus (UF), inferior longitudinal fasciculus (ILF), optic tract (Opt) in left and right hemispheres, and genu (GCC) and splenium (SCC) of the corpus callosum.

Statistical Analysis

Descriptive summary statistics were generated for the study parameters and their distributions evaluated to determine if transformations were needed. The 40 scans were categorized as being acquired from either continuously IS or ID monkeys based on their prior hematological values between 4 and 8 months of age (N = 26 and 14, respectively). MCV and Hgb values were compared by repeated measures analysis of variance to confirm the initial difference between IS and ID infants, and then to verify that the hematological values of previously ID infants had returned to the normal range of IS monkeys at the time of MRI scan. Following significant main effects for iron status and time, as well as a significant interaction term, pairwise *post hoc* comparisons were conducted with t-tests. The growth and brain parameters of anemic infants that received iron were also compared to the 3 untreated ones with moderate ID, and then all were combined into one group when found to be similar. Because of technical problems with data quality, one scan was excluded from both the volumetric and DTI analyses, resulting in a total of 14 scans for the previously ID monkeys.

Analyses of the volumetric brain data were conducted in three steps. Anemic status, including Hgb, size, age, and sex were considered as categorical variables and evaluated for their contribution to variance in TBV using a mixed linear model with and without interactions (with SPSS 25.0). Optimal independent variables were iron status, sex, and age. Then, analyses were run to evaluate TBV and GM and WM volumes in identical models, using ID as the predictor, and age at scan and sex as covariates. Finally, secondary analyses with the same models examined volumes in parcellated brain regions to assess if the effects were larger in specific regions. One rationale for the exploratory analyses related to sex was because male monkeys tend to be bigger with a larger head circumference. However, when including either TBV or ICV as a correction, it already adjusted for this sex difference in size. Females and males were similarly represented in both the IS and previously ID groups.

For the DTI analysis, a generalized linear model (GLM) was employed to analyze the effect of ID (i.e., binary variable) on WM maturation. Sex, age at time of scan, and weight at birth, as well as the interaction between sex and iron status were also included as shown below:

$$[\text{FA, AD, MD, RD}] = \beta_0 + \beta_1(\text{Sex}) + \beta_2(\text{Age}) + \beta_3(\text{BW}) + \beta_4(\text{ID}) + \beta_5(\text{ID}^*\text{Sex}).$$

The GLMs were tested first in an omnibus model that analyzed all 4 DTI properties jointly, followed by separate *post hoc* testing of each diffusion parameter. Statistical analyses were performed via FADTTSter (Noel et al., 2017), a tool for Functional Analysis of Diffusion Tensor Tracts Statistics (Zhu et al., 2011). The quality of the extracted fiber profiles were further assessed using minimal pairwise correlation with the average profile (see **Supplementary Table 3** for number of scans

[1] https://www.nitrc.org/projects/unc_macaque/

with high quality profiles; some outliers were excluded from the final analysis). Global omnibus *p*-values were corrected for the number of analyzed tracts using Bonferroni corrections (see **Supplementary Table 2** for detailed summary of test results). *Post hoc* analyses were performed for each DTI property to detect local effects within tracts that had evinced a significant effect of ID in the global analysis. Local *p*-values (uncorrected for multiple comparisons) were projected onto the fiber tracts for visualization of significant locations using 3D Slicer (version 4.8.1).

RESULTS

Hematological Values

Based on the occurrence of low MCV and Hgb values between 4 and 8 months of age, 15 of the 41 monkeys were classified as ID (**Figure 1**). Twelve met clinical criteria for anemia (MCV < 60 fL; HgB < 10 g/dL) and were administered iron dextran IM for a mean of 6 weeks (range 4–8 weeks) until their hematological values returned into the normal range. Three had a moderate ID and were not iron-treated, but were combined with the treated ID monkeys because their growth and brain data were similar. By 1 year of age, when the MRI scans were conducted, the hematological values of all previously ID monkeys, including the untreated ones, did not differ from MCV and Hgb values of monkeys who had remained continuously IS (**Figure 1**). Similarly, the body weights and head circumferences of IS and ID infants also did not differ significantly at the time of scans [body weight: 2371 (46) vs. 2278 (81) gm; head circumference: 24.6 cm (0.11) vs. 24.4 cm (0.23), IS vs. ID, respectively]. Males tended to be slightly larger than females (153.0 gm, $p < 0.051$) and had a larger head circumference (0.4 cm, $p < 0.03$) at 1 year of age, but both sexes were represented in the IS and previously ID groups. Because of quality issues with one scan, final analyses were conducted with the MRI and DTI data from 26 IS to 14 ID monkeys, excluding 1 iron-treated monkey.

Physical Growth

Analysis of the body weights at birth and then at 2-month intervals across the first year of life indicated there wasn't an overall main effect of iron status on overall growth. The birth weights of the IS and ID infants were similar and, as reported above, they were also of similar size at 10 and 12 months prior to acquiring the MRI scans. However, significant 2-way interactions between Age and Iron Status and Sex and Iron Status indicated there were selective differences between IS and ID monkeys at two age points (**Supplementary Table 4**). At 2 months of age, infants that would become ID were more likely to be larger, reflecting a stronger effect of rapid growth rate on iron utilization in male infants ($p < 0.03$). Conversely, infants that became ID tended to be smaller than IS infants by the end of the anemic period at 6 months of age (IS vs. ID, mean difference = 100 gm, $p < 0.071$). However, by 1 year of age when scanned, the previously ID infants were now of similar size, and there was only the trend for males to be larger than female monkeys (mean difference = 154 gm, $p < 0.068$).

Structural MRI

Total brain volume (TBV) and total GM volumes were significantly smaller in monkeys with a history of ID, and the difference was evident in both untreated and iron-treated monkeys (**Figure 2**). *Post hoc* analyses of GM volumes in the parcellated regions were conducted to determine if the effect of ID was global or more localized (**Table 1**). GM volumes in all regions except subcortical structures and cingulate were

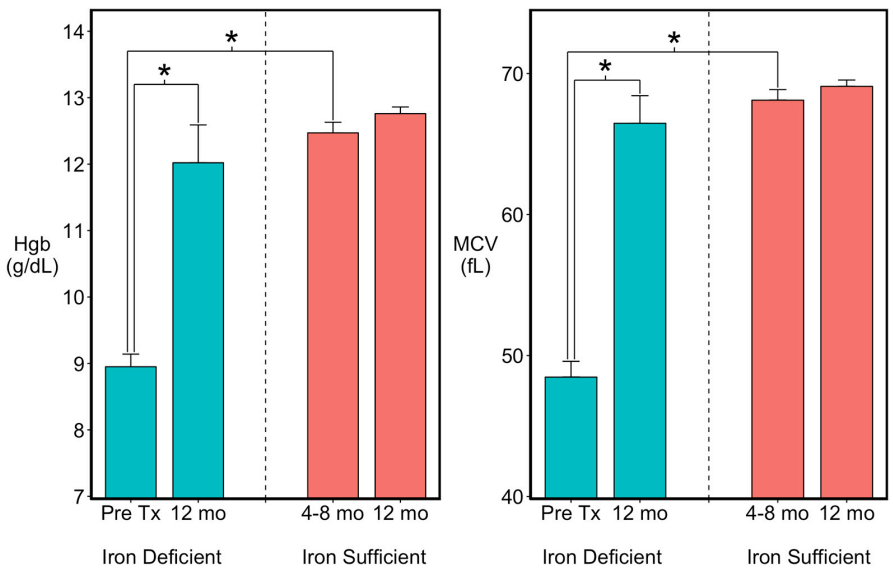

FIGURE 1 | Hematology of the infants categorized as previously iron deficient (ID) on the basis of lowest Hgb and MCV values between 4 and 8 months of age or as continuously iron sufficient (IS). At 1 year of age when MRI scans were acquired, the iron-related hematology of all infants was in the normal range. *$p < 0.05$.

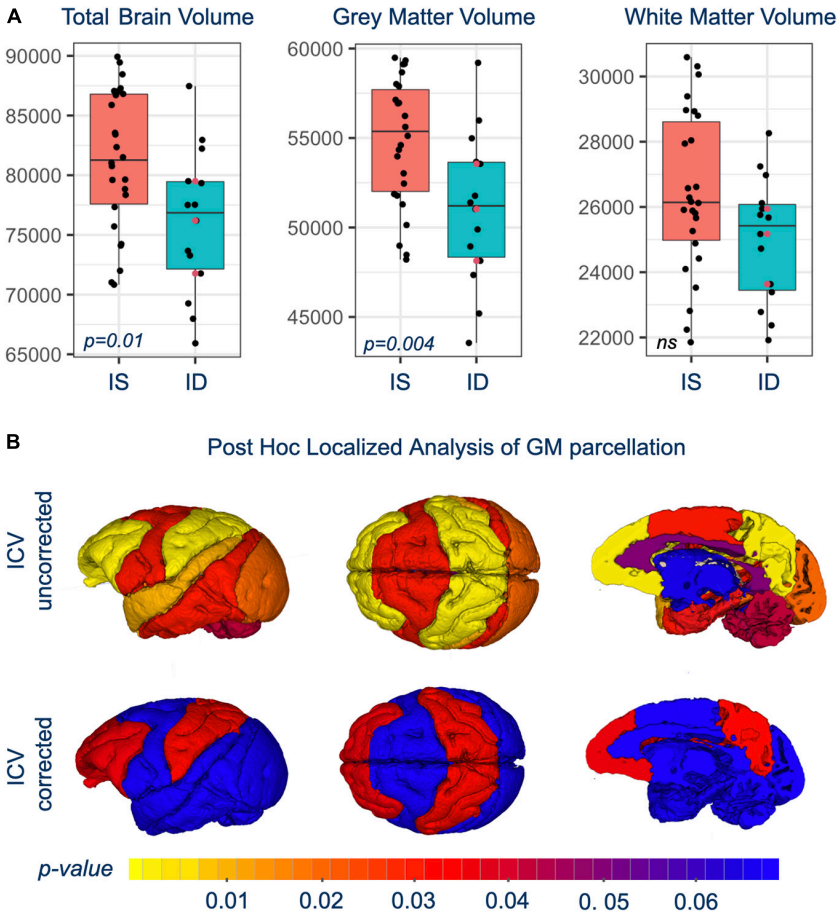

FIGURE 2 | (A) Shows box-and-whisker plots with mean difference in brain volumes between IS and previously ID infants. Red circles show the similarity of brain scan results for the untreated, moderate ID monkeys compared to the ID infants treated with iron dextran. **(B)** Is a visual representation of the significance of regional GM differences between IS and previously ID monkeys, without and with correction for ICV.

significantly smaller in monkeys with a prior history of ID. The regional GM values were also analyzed after correction for differences in ICV to further probe if there were larger localized effects in specific regions. This ICV-corrected analysis indicated that smaller GM volumes were most evident in the parietal and prefrontal cortices of the ID monkeys (**Table 1**). Despite the widespread effects of iron status on GM volumes, there were only statistical trends for global and regional differences in WM volumes at the macrostructural level and they did not reach significance.

Diffusion MRI

Even though there weren't overt macrostructural differences in WM, the global omnibus analysis of DTI values indicated the cingulum (CG) and uncinate fasciculus (UF) had been affected by a history of ID. Based on *post hoc* analyses, FA and RD parameters were the largest contributors to this effect of ID in the omnibus analysis (**Figure 3**). Monkeys in the ID group had lower FA values in left and right CG and UF, and a higher RD in the left and right CG and right UF, indicative of less integrity and myelin content. The differences appeared to be driven more by the female monkeys with a history of ID (**Figure 4**). Sex-related differences were evident in both hemispheres, and a significant interaction term between sex and magnitude of the ID effect suggested females had been more impacted. In addition, the sex- and ID-related differences were more pronounced in the right hemisphere after Bonferroni corrections for multiple comparisons. Finally, it should be mentioned that ID was not associated with less WM integrity in the two callosal tracts we analyzed (i.e., genu and splenium) or in the posterior tracts, including ILF and Optic tracts.

DISCUSSION

This analysis has demonstrated that a transient period of ID during infancy can significantly affect brain development in young monkeys. Finding that this effect persisted even after iron treatment, and months after hematological recovery, is in keeping with prior research documenting iron is essential for normal brain growth and that ID can have lasting effects on the developing brain (Georgieff, 2017; Wang et al., 2019). Iron is

TABLE 1 | Differences in total brain volume (TBV), and gray matter (GM), and white matter (WM) volumes between IS and ID monkeys.

Region	IS mean ± SE	ID mean (mm^3) ± SE	*p*-value ICV uncorrected	*p*-value ICV corrected
Global analysis				
TBV	81265 ± 1148	76043 ± 1639	**0.01****	**0.030**
GM	54836 ± 697	51046 ± 1145	**0.003****	**0.008****
WM	26429 ± 484	24997 ± 517	0.069	0.156
***Post hoc* localized analysis of GM after parcellation**				
Occipital	3941 ± 81	3624 ± 105	**0.021**	0.273
Temporal auditory	2073 ± 36	1909 ± 52	**0.007**	0.160
Subcortical	3049 ± 48	2900 ± 71	0.076	0.757
Frontal	3277 ± 50	3087 ± 76	**0.034**	0.959
Cerebellum	2607 ± 50	2437 ± 73	**0.049**	0.476
Insula	374 ± 9	342 ± 14	**0.018**	0.274
Cingulate	979 ± 20	916 ± 25	0.052	0.885
Parietal	3509 ± 51	3220 ± 85	**0.002****	**0.026**
Prefrontal	2980 ± 50	2731 ± 64	**0.002****	**0.028**
Temporal visual	3066 ± 47	2886 ± 79	**0.032**	0.631
Temporal limbic	1287 ± 19	1215 ± 33	**0.037**	0.588

*Post hoc contrasts for parcellated tissue volumes are shown both uncorrected and corrected for brain size as reflected by ICV. Significant group differences shown with and without correction for ICV. Bold font indicates p-values below 0.05. **p-values that remained significant after Bonferroni correction for number of models in this global analysis. Adjusted for sex and age at scan as covariates.*

needed to support neurogenesis, axonal growth, synaptogenesis and dendritic arborization during both the prenatal and postnatal periods. Using this primate model and the same diet, we had shown previously that the growth-related ID that first becomes evident in late infancy can affect oxidative energy metabolism in the periphery and in the CNS (Rao et al., 2018; Sandri et al., 2020). However, it was not known if iron treatment at the time of diagnosing anemia could prevent or lessen the persistence of brain effects. Despite rapid iron treatment, there continued to be a significant impact of ID on total brain size at 1 year of age. In addition to the global reduction in GM volume, focal effects were found in the prefrontal and parietal lobes. The effects on total and regional WM volumes were just statistical trends at the macrostructural level. However, at the microstructural level, there were significant differences in the DTI properties of two tracts, indicative of an effect of ID on myelin integrity in these later-maturing tracts.

Prior research on iron and brain development has highlighted the importance of iron for the synthesis of myelin. Therefore, we anticipated larger effects on WM volumes. But it is important to consider that the maturation of WM in monkeys extends out beyond the selected scan age of 1 year. Increases in WM are sustained through the pubertal transition at 3–4 years. Even when a genetically induced aberration in WM synthesis was investigated in a transgenic monkey model of Huntington's disease, the progressive deficits in WM became more evident later during the second year of life, with peak differences emerging in the third year (Meng et al., 2017). Both the timing of infantile ID and our scan age preceded this later period of WM maturation. At 1 year, significant effects of the prior ID were detected only with DTI in the uncinate fascicularis and cingulum. Future studies will have to determine if larger effects on WM volumes would be more apparent if the neuroimaging was repeated again at an older age. Alternatively, it is possible that there was sufficient plasticity and enough time for a recovery of myelin synthesis given that the ID lasted for only 2–3 months and resolved by 8 months of age with treatment.

Nevertheless, the persistent effects on GM volumes are still concerning. The brain of an infant monkey at birth is 60% of adult size and with rapid growth will reach 90% by 1 year (Knickmeyer et al., 2010; Scott et al., 2016). Thus, the large effect on GM volumes still present at 1 year could indicate that some of the neural differences will be sustained. That conclusion would be in keeping with previous metabolomic and proteomic analyses of CSF, which indicated the hypometabolic state of the CNS in ID monkeys may both precede and last beyond the period of hematological anemia (Rao et al., 2013, 2018). Similar findings on brain energetics have also been reported in rodent models of ID (Bastian et al., 2010, 2012).

In this monkey model, the emergence of ID first becomes clearly evident when the infant's growth-related needs for iron deplete prenatally acquired reserves and exceed the iron supply available in breast milk. It is not likely that the observed brain effects at 1 year were induced by limited iron in the fetus prior to birth. Even when pregnant monkeys consume a diet with lower iron levels, most infants typically have high ferritin levels at term, and even the ones that will become ID still have hematological values in the normal range until 2 months of age. However, we should acknowledge the importance of iron even for the fetal brain (Wang et al., 2019; McCann et al., 2020). It has also been shown that the levels of iron reserves at birth can be predictive of the risk for later anemia (Shao et al., 2020). Further, a neuroimaging assessment of human infants born to adolescent mothers who did not take sufficient iron while pregnant and were then scanned 2 weeks after delivery also found more prominent deficits in GM than WM (Monk et al., 2015).

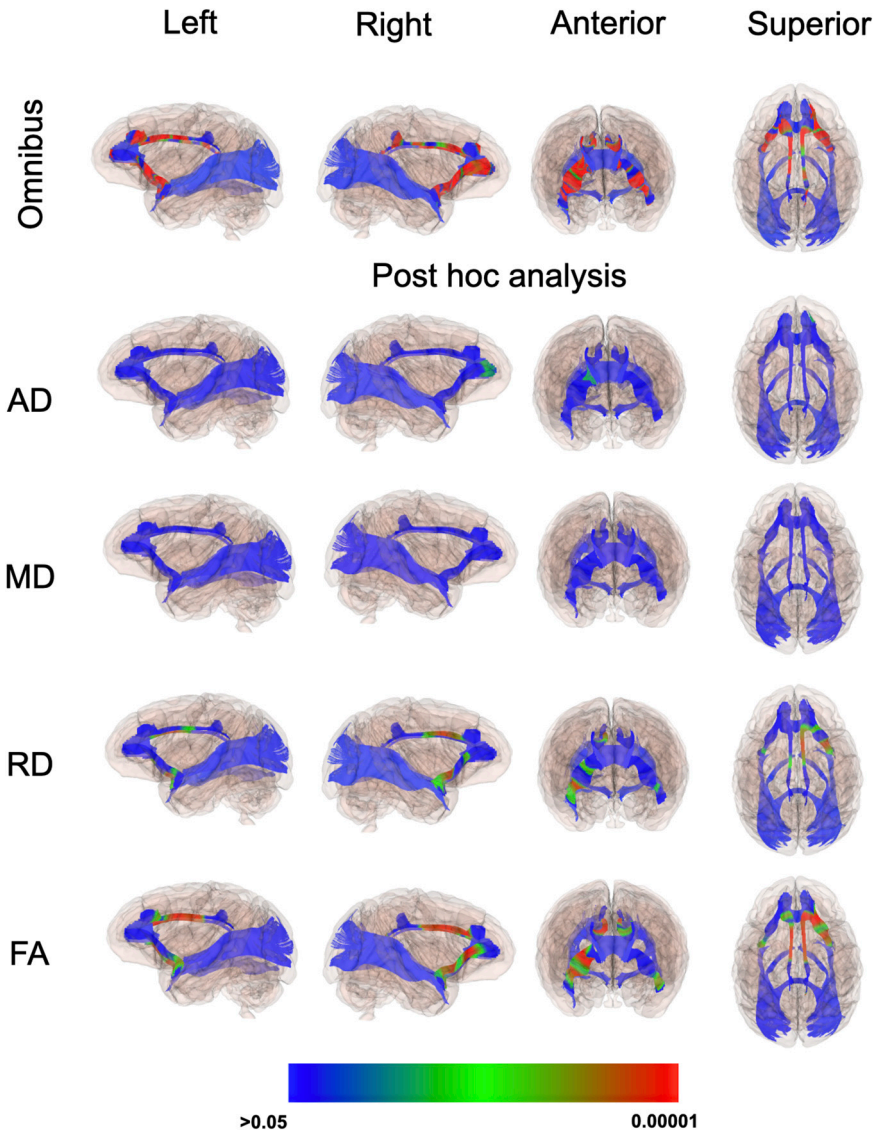

FIGURE 3 | *P*-value color scale illustrating the statistical significance of the local differences between IS and previously ID infants in the global omnibus analysis and separately for each of the four diffusion properties.

Relatively less GM in a rodent model of fetal/neonatal ID was attributed to truncated neurite outgrowth and reduced dendritic arborization (Bastian et al., 2016). Neurogenesis is extremely active during the late fetal stage (Gilmore et al., 2018), so it is possible that even small reductions in iron could have a long-lasting influence on axonal growth and synaptogenesis, which might then contribute to the later differences in GM volumes we found at 1 year.

It may also be of potential relevance that other neuroimaging studies of normal brain development in healthy monkeys without any diet manipulations detected a transient slowing of brain growth at weaning between 6 and 10 months of age (Scott et al., 2016). Thus, the occurrence of ID at this developmental transition to consuming solid food could have accentuated a normal deceleration in the rate of brain growth, which was then prolonged and still evident when scanned at 1 year. The larger effects on GM volume in the parietal lobe could reflect the overall size of this cortical region or a differential vulnerability because the maturing parietal cortex was also found to be particularly sensitive to the effects of maternal infection with influenza virus (Short et al., 2010). Growth of the parietal lobe is sustained postnatally and its connections extend during the developmental period when the ID occurred (Chow and Hutt, 1953; Knickmeyer et al., 2010; Scott et al., 2016; Sawiak et al., 2018).

Diffusion tensor imaging allowed us to look more closely at the microstructure of WM, and revealed bilateral reductions in FA in the cingulum and uncinate fascicularis. In addition, there were higher RD values in the same tracts, but only in the

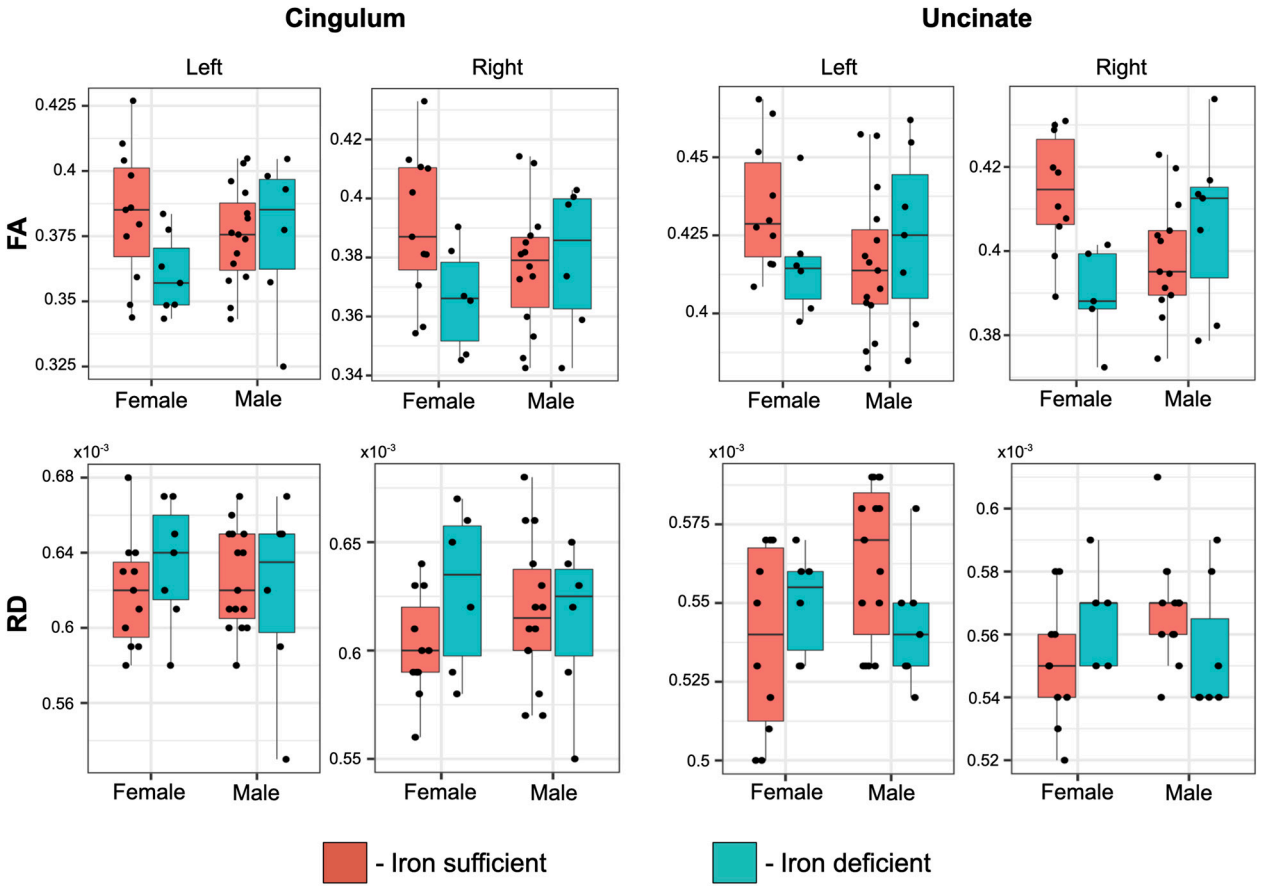

FIGURE 4 | Box plots of the effect of transient ID on FA and RD values in the cingulum and uncinate fascicularis, averaged per tract. The magnitude of the ID effect was significantly larger in females and more evident in the right hemisphere, resulting in a significant interaction between a prior history of ID and the monkey's sex.

right hemisphere. While FA is sensitive to the developmental pace of myelination, it is not specific to just maturation, and can reflect other processes, including fiber organization. RD has been more specifically linked to axonal myelination, as it reflects perpendicular diffusivity and the myelin sheath restricts diffusion in that direction (Alexander et al., 2007). These myelin differences were observed in ID infants whether they were rapidly treated or allowed to gradually recover via consumption of iron in food. In contrast, similar deficits were not evident in corpus callosal and posterior tracts, which develop earlier, likely reflecting the late onset of ID between 4 and 8 months of age. The observation of some effects on myelination is not a surprising finding. Iron is a required co-factor for lipid biosynthesis, and iron-rich oligodendrocytes are involved in the generation of the myelin sheath around the axon (Connor and Menzies, 1996; Todorich et al., 2009). Moreover, myelin synthesis is an energy-demanding process (Harris and Attwell, 2012), and energy-generating pathways are altered in the CNS of an anemic monkey (Rao et al., 2018).

Many experimental models of ID have focused more specifically on the vulnerability of the hippocampus (Pino et al., 2017). Our imaging results indicate there are additional effects on anterior tracts, which may help to explain some of the previously reported deficits in cognitive functions that rely on inter-hemispheric processing, as well as the effects on attentional and emotional processes. Those evaluations have indicated that some functional limitations persist even after iron therapy. One study found delayed auditory brainstem responses in children still evident at 12- and 18-month follow-ups (Roncagliolo et al., 1998). In animal models of ID, transferring rats from a low iron diet to a iron-sufficient one did not prevent the effect on axons in the optic nerve, including some structural deformation and smaller myelin sheath thickness (DeMaman et al., 2010). Similarly, with inbred mice strains that experienced a genetically programmed and time-delimited period of ID in the hippocampus, persistent transcriptomic and structural abnormalities were found to still be present in the adult hippocampus (Fretham et al., 2012; Barks et al., 2018).

It is important to emphasize that the experimental approach used to create this primate model of ID did not require any additional manipulations postnatally. Fifteen of 41 infants became ID as their growth-related needs for iron exceeded tissue reserves and the iron available in breastmilk. The prevalence of ID in the current study is consistent with previous experiments

that found approximately 20–30% became ID by 4–8 months of age (Coe et al., 2013). This percentage is also similar to the prevalence of infants who become anemic in low-income countries due to iron deficiency, after excluding other types of anemia that can be caused by parasitic infections and chronic disease during childhood (Armitage and Moretti, 2019). The path to ID during infancy is known to be multifactorial and includes large maternal weight gains during pregnancy, gestational diabetes, and hypertension, as well as the experience of maternal stress during pregnancy (Coe et al., 2007; Rendina et al., 2018). Collectively, it conveys the broader significance of iron biology for understanding how the effects of early life adversity are first initiated (Phillips et al., 2014).

When considering the translational relevance for public policy and the promotion of maternal and child health, it is important to reiterate that premature birth is still a common occurrence, especially in the context of adversity and poverty. Premature infants are much more likely to be iron deficient (Moreno-Fernandez et al., 2019). In addition, the later growth-related ID seen in the older infant monkeys is similar to what can occur in a subset of breastfed human infants, especially if exclusively breastfeeding past 6–12 months of age. Given that some effects on the brain may begin earlier and persist longer than hematological indices convey, it highlights the need to consider early pediatric screening of infants with known risk factors for anemia, especially after a premature birth (Baker et al., 2010). There may also be benefits of preemptive iron supplementation for a rapidly growing baby who is not fed iron-fortified formula or provided with some solid foods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the University of Wisconsin.

AUTHOR CONTRIBUTIONS

RV, MS, PK, MG, RR, GL, and CC were involved in the funded project. QW and AW contributed to the analysis of the MRI data and writing. RV and QW worked together to analyze the volumetric and DTI results. All authors have reviewed several manuscript drafts and the final version of the 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/fnhum.2021.624107/full#supplementary-material

Supplementary Table 1 | Ingredient composition, as well as mineral and vitamin concentrations, for the standard diet fed to the pregnant and nursing mothers and then to the juvenile monkeys after weaning in this study[1]. [1]LabDiet, 5LFD. [2]Based on typical daily consumption (200 g) the adult female monkeys were provided approximately 45.0 mg Fe from the biscuit diet each day.

Supplementary Table 2 | Diffusion MRI omnibus global *p*-values. Diffusion global omnibus *p*-values for Left and Right Hemisphere (cont.). **p*-value remained significant after Bonferroni correction for number of tracts.

Supplementary Table 3 | Number of subjects remaining in the analysis after profile QC.

Supplementary Table 4 | Body weights (gm) of infant monkeys at 2-month intervals from birth through one year of age when the MRI scans were acquired. Mean values (+SE) shown for males and females, as well as for the monkeys that remained continuously iron sufficient (IS) or became transiently iron deficient (ID). *Significant main effect of age indicative of normal growth, $p < 0.001$. **Significant main effect of sex, with mean weight for males larger than females, $p < 0.05$. ‡Mean weights for IS and ID infants did not differ. However, 2-way interactions between Age and Iron Status, and Sex and Iron Status in the ANOVA followed by *post hoc* testing indicated the weights of IS and ID infants differed at 2 time points. At 2 months of age, male ID infants were the largest ($p < 0.03$), whereas at 6 months the ID monkeys tended to be somewhat smaller after the period of anemia than IS monkeys ($p < 0.08$). @Weights at 10 months and then at 12 months of age when the MRI scans were acquired did not differ between IS and ID. The hematology of the previously ID monkeys was also in the normal range at the time of the scans.

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Prenatal Relocation Stress Enhances Resilience Under Challenge in Infant Rhesus Macaques

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The prenatal period is a developmental stage of peak sensitivity, during which environmental exposures can program post-natal developmental outcomes. Prenatal stress, in particular, has often been associated with detrimental neurobehavioral outcomes like mood and anxiety disorders. In the present study, we examined the effects of a stressful prenatal maternal experience (maternal relocation during pregnancy) on the post-partum development of offspring in rhesus macaques. To help isolate the effects of prenatal stress from genetic predispositions and post-natal experience, we compared biologically reared infants (infants raised with their biological mothers) with cross-fostered infants (those raised by non-related females in new social groups). We examined the effects of prenatal relocation stress on measures collected at 3–4 months of age during a standardized biobehavioral assessment. Unexpectedly, we found that prenatal stress resulted in a behavioral pattern consistent with resilience rather than anxiety: prenatal stress was linked with greater activity, lower anxiety, and more interaction with novel objects, as well as higher ratings of temperamental confidence during assessment. These effects were observed in infants reared by biological mothers as well as cross-fostered infants, suggesting that the effects of prenatal stress were not attributable to maternal genetics or post-natal factors. Our surprising results suggest that prenatal relocation stress may confer resilience in infant rhesus monkeys.

Keywords: prenatal stress, infant behavior, resilience, temperament, rhesus monkey

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INTRODUCTION

Prenatal Stress and Functional Dysregulation

The prenatal period is a peak sensitivity period during which teratogens like drugs, alcohol, and stress, can shift infant development toward disadvantageous outcomes (Laegreid et al., 1989; Schneider et al., 2002; Ruiz and Avant, 2005). Maternal stress during pregnancy has been found to cause pro-inflammatory and stress axis changes that may permeate the placenta and reach the developing fetus (Ruiz and Avant, 2005). Chronic stress has also been observed to cause an over stimulation of the maternal hypothalamic-pituitary adrenal (HPA) axis, which results in excessive amounts of glucocorticoid production that can also cross the placenta barrier and potentially lead to emotional, intellectual, and neurodevelopment deficits (Gunnar, 1998). Some have reported that intense maternal prenatal stress, such as disruptive social changes, unfavorable living conditions, or exposure to substance abuse confers risk for behavioral and temperamental deficiencies like

increased emotional reactivity, reduced exploration of the environment, and anxiety (Schneider et al., 2002; Richardson et al., 2006; Lester et al., 2009; Herrington et al., 2016). Others have demonstrated that prenatal stress can foster risk for externalizing behavior as children mature. For instance, Gutteling et al. (2005) found that mothers who reported a greater amount of perceived stress during pregnancy had toddlers who showed more problematic externalizing behavior, had greater amounts of disruptive temperament, and poorer attention regulation.

In contrast, some prenatal stressors seem to "inoculate" the developing infants against the harmful effects of stress by promoting resilience (DiPietro et al., 2006; Hartman et al., 2018; Serpeloni et al., 2019). DiPietro et al. (2006) showed that mild to moderate amounts of perceived stress and anxiety by the mother during pregnancy were associated with more advanced mental and motor development in human children at 2 years of age. A more recent report showed that prenatal stress may confer a protective phenotype, buffering animals against future stressors (Scott et al., 2017). Pregnant female mice were exposed to a series of chronic variable stressors during their last week of pregnancy. As adults, the prenatally stressed male offspring were housed in a naturalistic environment, with control male, and female counterparts. Prenatally stressed males were more often observed to occupy a subordinate social status as opposed to a dominant status, but the detrimental effects (significant weight loss, behavior inhibition: fleeing, hiding, incurring more wounds) associated with a subordinate status in control males were not observed. These data suggest that prenatal stress may promote resilience to social subordination stress in mice.

Some of the variability in the effects of prenatal stress in human studies may be attributed to complex interactions between prenatal stress and post-natal stress: prenatal socioeconomic status (Lobel et al., 1992), support systems (Dunkel-Schetter et al., 1996), or maternal attributes (DiPietro et al., 2006) each contribute to both the prenatal and post-natal environment. On the other hand, the experience of a prenatal stressor may change the post-natal environment a mother may provide. Animal models allow us to isolate genetic, prenatal, and post-natal influences on individuals through *cross fostering* infants to unrelated mothers in new social groups (Champoux et al., 1995; Francis et al., 1999; Kinnally et al., 2018). Non-human primates exhibit comparable reproduction and gestational characteristics to that of humans but in a reduced time frame making them excellent candidates for longitudinal studies (Schneider et al., 2002). Rhesus monkeys (*Macaca mulatta*) make an exceptional translational animal model of development due to their phylogenetic similarity to humans (Phillips et al., 2014).

Current Study

The purpose of our study is to examine the effects of stressful prenatal events experienced by pregnant macaque females on the post-natal development of their offspring. Previous studies in macaques have examined the effects of other prenatal stressors (matrilineal overthrow: Herrington et al., 2016; chronic relocation with acoustic startle: Coe et al., 2003). Our opportunistic model of stress utilizes hospital relocations due to: injury, illness, or offspring related reasons for which the animal is temporarily removed from their home environment for an average of 9 days over an average of 1.5 relocations during pregnancy. This temporary relocation is a stressful, but common, event for outdoor housed captive rhesus macaques. Relocation requires temporary separation from family and peers and introduction into a novel environment, a procedure that animals will experience many times throughout their life course (Capitanio and Lerche, 1998; Dettmer et al., 2012; Hennessy et al., 2017). Permanent relocation to new social groups has been shown to increase HPA output and self-injurious behavior in the short- and long-term [1 year later: Davenport et al. (2008)]. Monkeys infected with SIV virus that are relocated frequently on a short-term basis exhibited decreased survival, perhaps resulting from this stress (Capitanio and Lerche, 1998). Most similarly to our short- term relocation model, Hennessy et al. (2014), observed a temporary, reversible depressive-like response in rhesus macaque adult males when relocated from outdoor cage housing to indoor individual housing for an 8-day period (Hennessy et al., 2014, 2017). Our study examines the association amongst exposure to intermittent short- term relocation stress during females' pregnancy and post-natal offspring behavioral development. We further investigated the role of genetic and post-natal factors in the effects of prenatal stress by comparing development in infants that remained with biological mothers with infants that were cross fostered to unrelated mothers in new social groups.

METHODS

Experimental Subjects

One hundred eighty-eight infant rhesus macaques (89 males and 99 females) were raised with their biological or foster mothers in one of seven social groups housed in large outdoor enclosures that constitute the breeding colony of the California National Primate Research Center (CNPRC). Each field cage contained a large social group (80–150 members). These groups had similar social demographics, including 6–13 distinct matrilines with extended kin networks and animals of all age/sex classes. A subset of our animals ($N = 57$) were raised in specific pathogen free (SPF) enclosures. These groups were free of zoonotic diseases such as Herpes B. These groups had been originally founded with mostly nursery reared monkeys [for review, see Kinnally et al. (2018)]. However, all of our groups were intact for at least 3 years at the time of observation and on average, established 15 years prior to observation, so most of our subjects had themselves been born and raised in SPF enclosures, similar to non-SPF females. Eight of our SPF mothers had been nursery reared and 49 had been born and raised in our outdoor enclosures.

All social groups included 5–10 reproductively mature males, 25–60 reproductively mature females, and 25–75 subadult, juvenile or infant monkeys. Enclosures were 0.2 hectares with chain link fencing to allow visual access. Animals were fed monkey chow (LabDiet 5405) twice per day, once in the morning and once in the afternoon. All groups received fresh produce on a weekly basis. All procedures were approved by the UC

Davis Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Prenatal Relocation

We retrospectively extracted relocation data from CNPRC health records. Conception day was defined as 166 days (the macaque gestation period, Silk et al., 1993) before infant birth. All relocations of a pregnant female from the date of conception through date of infant birth were extracted. Relocations were defined as transitions from outdoor natal enclosures to indoor individual housing for a total of at least 24 h. There were three possible reasons females were relocated during pregnancy: injury, illness, or hospitalization of their current infant. Relocation trimester was calculated as: first trimester 0–55 days, the second trimester 55–110 days, and the third trimester 110–166 days. During relocation, the most common pharmacological treatments were ketamine (a sedative) and ketoprofen (a non-steroidal anti-inflammatory). Relocation lengths consisted of 1–36 days across 1–5 relocation bouts. Mean length of relocation days across all of the relocations was 9.25 days and the average number of relocations was 1.48 relocations. We used a dichotomous category for relocation because statistical analyses revealed that neither length, nor number of relocations, nor trimester of relocation, moderated the main effect of any relocation during pregnancy. Our prenatal stress exposure was therefore defined experiencing any relocation during pregnancy ($N = 39$), compared with females that were not relocated at any time during pregnancy ($N = 149$). See **Table 1** for demographic comparisons between prenatal stress and control groups.

Cross-Fostering

Cross-fosters were selected to enhance genetic diversity between social groups. Post-partum mothers were identified on the morning of birth, and selected if they: (1) were unrelated to potential cross-foster mothers in other social groups, (2) had previously successfully reared a foster infant (all but one female adhered to this criterion) (3) had previously given birth to at least one infant. On post-natal Day 1, mother-infant dyads either remained undisturbed in outdoor enclosures ($n = 146$) or were relocated indoors for standardized cross fostering procedures ($n = 42$) that we have described in detail elsewhere (Kinnally et al., 2018, 2019). Briefly, dyads were removed from outdoor enclosures and transported to indoor procedure rooms. Mothers were sedated (10 mg/kg ketamine) and infants were removed by trained CNPRC staff and placed on the ventrum of the new foster mother who was also sedated. Dyads were monitored overnight to ensure that the foster mother accepted the infant after manipulation. Success of our cross-fostering procedure was 100%, with all foster mothers accepting new infants and returning to social groups within 24 h. Of our prenatal stress group, nine infants were foster reared and thirty were biologically reared. In our control group, thirty- three infants were foster reared and one hundred sixteen were biologically reared. Of our foster mothers, six had been prenatally relocated. Five of these mothers fostered offspring that had not been prenatally relocated and one fostered an infant which had been prenatally relocated.

Mother-Infant Observations

Mother-infant dyads were observed in their social groups for 5 min per observation using focal dyadic sampling (Altmann, 1974), conducted one to four times weekly (range 1.25–4.25, average 2.27 observations per week), as we have described previously (Kinnally, 2014; Kinnally et al., 2018, 2019). Observations were conducted between 7:00 A.M. and 1:00 P.M., during post-natal weeks 1–12. Observation order was rotated daily so dyads were observed at different times of day across the prescribed observation period.

Mother-infant interactions were coded using a transactional coding system, describing the overall theme of an interaction from the perspectives of the initiator and the recipient (Lyons-Ruth et al., 1986; Kinnally et al., 2010; Kinnally, 2014). A transaction was defined as a change from one state of association that lasted 3 s or more, to a new state that was maintained for at least 3 s. Themes are defined in **Table 2**, and include protection, affiliation, neutral, rejection and aggression. Each dyadic interaction was characterized from the perspective of the mother and of the infant, and a theme assigned for each based on their behavior. For example, some common transaction types include: (1) infant approaches mother and initiates contact, mother initiates physical contact (which would be scored as Affiliative-Affiliative, received by the mother), (2) Mother retrieves infant and infant grabs mother's ventrum or back (Protective-affiliative), (3) Infant jumps on mother, mother swats the infant to the ground (Affiliative-aggressive, received by the mother).

Adult raters were trained by a primatologist with experience in mother-infant interactions for at least eight 2-h training sessions. Rater reliability was determined at the end of the training period by calculating whether the rater was correct in recording each aspect of the transaction (maternal theme, infant theme, and recipient) for ten sequential 5-min trials. These trials were required to comprise of a wide variety of transaction types or were otherwise excluded from reliability calculations. Inter-rater reliability was 90% or better.

Rates of all transaction themes by the mother (Protective, Affiliative, Neutral, Rejecting or Aggressive) were calculated per observation period for analysis as we have described previously (Kinnally et al., 2010, 2018, 2019; Kinnally, 2014).

Biobehavioral Assessment

At 90–126 days of age (mean 99.88 days), subjects were observed during a standardized biobehavioral assessment (BBA) at the CNPRC. During a 25-h relocation and separation from mothers and social groups, multiple behavioral (activity, emotionality, novel object interaction, temperament ratings), and physiological (plasma cortisol at four time points, selected to minimize impact on behavioral measures) measures were collected from infant subjects. The purpose of the biobehavioral assessment is to gauge infant individual differences under challenge conditions, when their mothers are not present to shape their behavior. The goal is to tap into the infant's biobehavioral organization—the structure of an individual's physiological and behavioral phenotype—that is intrinsic to them. Standardized procedures were designed to ensure that each subject had experiences comparable to all other

subjects who underwent assessment. These procedures have been described in detail elsewhere (Golub et al., 2009; Kinnally et al., 2010; Capitanio, 2017). Standardized procedures included exposure to a single observer, both between tests and between years. The technician was unfamiliar with the animals before their arrival in our testing area for the BBA.

Holding Cage Observations

Subjects were relocated from their outdoor enclosures to indoor individual housing (0.81 × 0.61 × 0.66 m) in a temperature-controlled room under a 12:12 h light/dark cycle by 9:00 a.m. At ∼9:15 a.m., animals' activity- and emotion-related behaviors were recorded in a 5-min trial (see **Table 3** for Ethogram). Two scales were identified using exploratory and confirmatory factor analyses on various sample subsets for these data from Day 1, as described in Golub et al. (2009). Factor analysis is a useful technique for identifying latent dimensions underlying multiple behaviors within or across contexts. Exploratory factor analysis identifies the structure of relationships between behaviors by assigning factor loadings. Factors that load higher than 0.4 are considered to contribute to the final factor score, which uses regression methods with factor loadings as beta weights to generate one factor score, which is a standardized score, or Z-score. Confirmatory factor analysis applies this structure to determine goodness of fit in replication

TABLE 1 | Prenatal stress vs. control female demographics.

	PS ($n = 39$)	Control ($n = 149$)	p
Age	6.613	6.531	0.718
Prior pregnancies	2.260	2.210	0.533
Rank (rank/#females in group)	0.484	0.537	0.532
Maternal affiliation	0.303	−0.079	0.080
Maternal aggression	0.006	0.008	0.823
Maternal protectiveness	−0.257	0.104	0.160
Maternal neutrality	0.101	−0.053	0.245
Maternal sensitivity	0.088	−0.022	0.411
Number of days relocated	9.25	0.00	0.000
Reason for relocation (PS only)			
Illness—mother	4	NA	NA
Illness—infant	1	NA	NA
Injury—mother	34	NA	NA
Ketamine exposure	3.415	1.012	> 0.0001
Ketoprofen exposure	0.293	0.006	> 0.0001
Infant birth weight	0.986	0.936	0.100

TABLE 3 | Home cage and human intruder ethogram.

States

SI, sit: Hindquarters are on the floor; includes shifting weight slightly one step.

LI, lie: Relaxed posture with body resting on a horizontal surface.

ST, stand: Torso in a stationary position and weight is supported by 3 or 4 legs; can include steps taken that only involve one or two steps.

LO, locomote: Directed movement from one location to another.

CR, crouch: Ventral surface close to floor; head at or below shoulders.

SL, sleep: Eyes closed.

PA, pace: Repetitive rapid movement over the same path.

RO, rock/sway: Unbroken rhythmic movements.

HA, hang: Holding onto ceiling, sides or front of the cage; all 4 limbs off of floor.

Events

SC, scratch: Common usage.

CL, self-clasp: Hand or feet closed on fur or some body part.

SB, self-bite: Discrete biting action, usually directed to limbs.

SS, stroke: Gently bringing the hand or foot across the side of head or face.

SU, suck: Insertion into mouth of fingers, toes, and other body parts.

BF, back-flip: Tossing the body up and backwards in a circular motion in the air.

CJ, convulsive jerk: Sudden contractions of the limbs and trunk.

VC, coo: Medium-pitched, moderately intense, clear call.

VS, screech: Intense, very high-pitched.

VG, gecker: Staccato cackling sounds.

VB, bark: Gruff, abrupt, low-pitched vocalization.

LS, lip smack: Rapid lip movement usually with pursed lips, smacking sound.

TH, threat: Two or more of the following: open mouth stare, head bob, ear flaps, bark vocalizations.

FG, fear grimace: Exaggerated grin with teeth showing.

YA, yawn: Wide open mouth displaying teeth.

TG, tooth grind: Loud gnashing of teeth.

MS, motor stereotypy. Any of the following: Repeated movement, of a head flip, sway (side to side motion while standing or hanging), or up and down motion of the body.

EE, Environmental explore: Discrete manipulation by hand or mouth with the physical environment or objects in the cage.

ET, eat: Common usage.

DR, drink: Common usage.

TABLE 2 | Maternal behavior transaction theme definitions.

Protective	Includes all behaviors intended to protect and restrict the infant's range of movement. The defining characteristic is the action of the mother pulling the infant toward her. Protectiveness is the only transaction that infants cannot engage in.
Affiliative	Includes behaviors including prosocial physical contact, such as grooming, licking, holding, and any other non-aggressive positive touch.
Neutral	Includes non-committal behaviors. The most common neutral transaction initiation is an approach without making physical contact. Neutral responses are those where the receiver does not react positively or negatively to an overture.
Rejecting	Includes behaviors that discourage interaction. The most common rejection theme includes walking away when mother approaches. This theme can only be a response to an overture.
Aggressive	Includes physical contact aggression. Includes scratching, hitting, biting, flattening, dragging, throwing, and any other physical contact that may inflict pain on the infant.

datasets. Holding cage scales were labeled Activity because they loaded higher frequencies or durations of locomotion, eating, drinking, environmental exploration, crouching, and less hanging. Emotionality was so-named due to the high loadings of cooing, barking, scratching, threatening, and lipsmacking. Z-scores within year are used as outcome variables to control for year-to-year variation.

Human Intruder Trials

At ∼2:00 p.m., each animal was tested in a single session comprising 4 1-min trials in a separate room from holding cages. The first trial ("Profile-Far") involved the experimenter positioning herself 1 m in front of the animal's cage, and presenting her left profile to the animal for 1-min. At the end of the minute, the experimenter moved laterally toward the animal's cage, positioning herself to within 0.3 m from the front of the animal's cage, still holding the profile position ("Profile-Near"). One minute later, the experimenter returned to the 1 m location and made direct eye contact with the animal ("Stare-Far" condition). After 1 min, the experimenter moved to the near (0.3 m) position while maintaining eye contact. This position was also held for 1-min ("Stare-Near"). The same experimenter was used for all animals. Preliminary analysis showed that the best strategy for dealing with the four conditions that characterize this test (e.g., profile far, profile near, etc.) was to take a mean for each behavior across the four conditions. Exploratory and confirmatory factor analyses were done, and a four-factor solution was found (Gottlieb and Capitanio, 2013): Activity (active, cageshake, environment explore), Emotionality (convulsive jerk, fear grimace, self-clasp, vocal), Aggression (threat, vocal bark, vocal other), and Displacement (tooth grind, yawn). Z-scores within year are used at outcome variables.

Novel Object Interaction

For the duration of the biobehavioral assessment, one of two small objects were present in the animal's housing at all times. Inside these objects was a recording device (Actiwatch, Philips Respionics, Andover, MA, USA) which is activated whenever a force is exerted on the object. First, a small (3.5″L × 1.5″D) black cylindrical shaped object weighing 0.090 kg, was placed in the infant's cage from the time the animal was relocated at 9:00 A.M. At ∼4:15 P.M., a second white crayon shaped novel object of similar size (3.6″L × 1.5″D) weighing 0.085 kg and with the same Actiwatch recorder, was placed in the holding cage and remained until the end of assessment at 9:15 A.M. the following morning. Data from the recorders were parsed into the number of 15-s intervals for each 5-min period during which any force was exerted on the objects. Period 1 includes the time block from ∼9:00 A.M to 12:15 P.M. on Day 1. Period 2 was defined as approximately the time between 12:15 P.M. and 4:30 P.M. Period 3 is defined as the overnight period between ∼4:30 P.M. and 8:00 A.M. the next morning. Period 4 includes the time between 8:00 A.M. and 10:15 A.M.

Temperament Ratings

Temperament data were collected by a trained observer at the end of the 25-h testing. The rater recorded her perception of each subject with regard to 16 adjectives describing temperament (e.g., confident, nervous, tense, timid), on a Likert scale of 1–7. Temperament assessments were performed by the same technician who had tested the animals in all of the other assessments. The technician specifically does not rate the animals until some time has elapsed between her last behavioral data collection and the ratings, in order to avoid having a recency effect on her memory of the animals. Because the temperament assessments are designed to reflect the technician's total experience with the animals during the 25-h period (i.e., not just while testing the animals individually, but also while handling during blood collection, performing husbandry procedures, etc.), getting inter-observer reliability on a regular basis is difficult, necessitating a second person accompanying the main technician across the entire testing period. When we have done this, we have found acceptable levels of inter- rater agreement and reliability. Finally, we note the values for the temperament measures are z-scored within each observation year, which can serve to minimize bias associated with technician "drift" over the years. We do note that the technician is highly experienced, having assessed all animals ($n > 5{,}000$) since the beginning of the BBA program in 2001, and has been involved with other studies that used a similar rating methodology. Exploratory and confirmatory factor analyses were applied to scores (Golub et al., 2009) on each of these adjectives to detect underlying dimensions of temperament. Four dimensions were found, which include Nervousness (including high scores on nervous, fearful, timid, and low scores on calm, confident), Confident (including high scores on confident, bold, active, curious, playful), Gentle (including high scores on gentle, calm, flexible, curious), and Vigilant (including high scores on vigilant, and low scores on depressed, tense, timid). Z-scores within year are used at outcome variables.

Plasma Cortisol

Procedures for blood sampling and drug treatment have been described in detail previously (Capitanio et al., 2005). Blood was sampled through femoral venipuncture four times over a 24-h period, and each sample was decanted into ethylenediaminetetraacetic acid-(EDTA) treated collection vials. The first sample was collected at 11:00 A.M. (AM sample), ∼2.5 h following social separation/relocation. The second blood sample was collected ∼5.0 h after the first sample at 4:00 P.M. (PM sample). Subjects were then immediately injected intramuscularly with dexamethasone (500 mg/kg; American Regent Laboratories, Shirley, NY, USA). The next blood sample was taken through femoral venipuncture 16.5 h later, at 8:30 A.M. (DEX sample). Immediately following blood sampling animals were injected with 2.5 IU ACTH (i.m. Organon, Inc., West Orange, NJ) and the final blood sample was collected 30 min later. Plasma cortisol was assayed in duplicate using commercially available kits (Diagnostics Products Corporation, Los Angeles, CA, USA). Interassay and intraassay coefficients of variance

(calculated as the SD of relevant samples/mean of relevant samples) were < 10%.

Data Analysis

Correlations among behavioral and physiological measures were conducted using Pearson's correlations. We conducted one multivariate analysis of variance (MANOVA) with sixteen dependent behavioral variables: four temperament factor scores (Vigilance, Confidence, Gentleness, Nervousness), holding cage Activity and Emotionality scores on Days 1 and 2, Novel object interaction during 4 binned time periods, human intruder factor scores (Activity, Emotionality, Aggression, and Displacement). We conducted an additional MANOVA with four dependent physiological variables: Predictors included prenatal relocation (present or absent) and foster status (cross-fostered or biologically reared). Covariates included maternal age, the mother's number of previous pregnancies, maternal social hierarchy rank (absolute rank divided by the total number of adult females in the social group), infant sex, and four maternal care factors: maternal protectiveness, affiliation, neutrality, and aggression. Roy's Largest Root was used for reporting multivariate tests. *Post-hoc* testing for individual dependent variables was conducted using individual F-tests.

RESULTS

The omnibus MANOVA model showed that maternal relocation during pregnancy significantly predicted biobehavioral organization across each of our assessments [Roy's Largest Root = 0.173, power = 0.888, $F_{(16, 174)} = 1.722$, $p = 0.047$, partial eta$^2 = 0.148$]. Prenatally stressed infants exhibited higher Activity scores on Day 1 of assessment [$F_{(16, 174)} = 3.898$, $p = 0.05$, partial eta$^2 = 0.022$] as well as Day 2 of assessment [$F_{(16, 174)} = 4.380$, $p = 0.036$, partial eta$^2 = 0.025$], in comparison to counterparts whose mothers had not been relocated during pregnancy (see **Figure 1**). Prenatal relocation was also associated with lower Displacement scores in the human intruder test [$F_{(16, 174)} = 4.414$, $p = 0.037$, partial eta$^2 = 0.025$; see **Figure 2**]. Prenatally relocated offspring also exhibited significantly higher rates of interaction with a novel object during period 2 [$F_{(16, 174)} = 5.35$, $p = 0.022$, partial eta$^2 = 0.03$], and period 3 of assessment [$F_{(16, 174)} = 4.086$, $p = 0.045$, partial eta$^2 = 0.023$; see **Figure 3**]. Prenatal relocation was also linked with higher Confidence scores at the trend level [$F_{(16, 174)} = 3.713$, $p = 0.056$, partial eta$^2 = 0.021$; see **Figure 4**]. Foster status was not related to any measure [$F_{(16, 174)} = 0.541$, $p = 0.922$, partial eta$^2 = 0.05$], as a main effect or in interactions with prenatal stress [$F_{(14, 173)} = 0.866$, $p = 0.609$, partial eta$^2 = 0.081$; see **Figure 5**].

No significant differences in biological vs. foster mothers were observed in any maternal behavior measure (MANOVA F = 1.089, $p = 0.363$).

Of all maternal and infant factors considered in our model, only maternal rank [Roy's Largest Root = 0.164, $F_{(16, 174)} = 1.948$, $p = 0.020$, partial eta$^2 = 0.164$] and SPF status [Roy's Largest Root = 0.187, $F_{(16, 174)} = 1.858$, $p = 0.028$, partial eta$^2 = 0.158$] predicted overall biobehavioral organization. The effects were such that offspring of lower ranked females exhibited significantly greater Activity on Day 1 [$F_{(1, 174)} = 5.379$, $p = 0.022$, partial eta$^2 = 0.030$], greater Emotionality on Day 1 [$F_{(16, 174)} = 7.455$, $p = 0.007$, partial eta$^2 = 0.041$], and greater Emotionality on Day 2 [$F_{(16, 174)} = 4.29$, $p = 0.04$, partial eta$^2 = 0.024$]. Lower ranked females had offspring that were rated as more vigilant [$F_{(16, 174)} = 4.216$, $p = 0.042$, partial eta$^2 = 0.024$]. SPF infants were significantly less active on Day 1 [$F_{(16, 174)} = 7.729$, $p = 0.006$, partial eta$^2 = 0.043$], and interacted with a novel object less often during Period 2 [$F_{(16, 174)} = 10.234$, $p = 0.002$, partial eta$^2 = 0.056$], Period 3 [$F_{(16, 174)} = 6.391$, $p = 0.012$, partial eta$^2 = 0.035$], and Period 4 [$F_{(16, 174)} = 4.396$, $p = 0.037$, partial eta$^2 = 0.025$]. SPF infants exhibited higher Emotionality [$F_{(16, 174)} = 4.445$, $p = 0.036$, partial eta$^2 = 0.025$] and Aggression scores [$F_{(16, 174)} = 4.04$, $p = 0.046$, partial eta$^2 = 0.023$] in response to a human intruder. *Post-hoc* testing revealed that the only maternal behavior factor that predicted infant behavior was Neutral mothering. Infants that received higher rates of neutral overtures and response from mothers were more active on Day 1 [$F_{(16, 174)} = 6.47$, $p = 0.011$, partial eta$^2 = 0.036$] and more gentle [$F_{(16, 174)} = 7.407$, $p = 0.007$, partial eta$^2 = 0.041$]. Infants of prenatally relocated mothers did not differ in birth weight ($t = 1.635$, $p = 0.100$), and means of prenatally relocated infants were on average higher (0.986 vs. 936 kg, respectively).

Though our omnibus model did not reveal an overall effect of relocation on infant cortisol [$F_{(1, 171)} = 1.381$, $p = 0.243$], *post-hoc* tests revealed that prenatal stress was associated with lower cortisol both in the post-stress samples [$F_{(1, 171)} = 4.094$, $p = 0.045$] and at the trend level post-ACTH [$F_{(1, 171)} = 3.429$, $p = 0.066$; see **Figure 6**].

Biobehavioral measures across conditions were moderately correlated, as we have previously reported (Kinnally et al., 2010; **Supplementary Table 1**).

DISCUSSION

The consequences of prenatal stress on infant development are diverse (Clarke and Schneider, 1993; Gutteling et al., 2005; DiPietro et al., 2006; Hartman et al., 2018). Some studies have shown prenatal stress leads to internalizing outcomes (negative affect, poor soothability, anxiety; Gutteling et al., 2005; Hentges et al., 2019), some to externalizing outcomes (impulsivity, poor self control; Gutteling et al., 2005; Betts et al., 2014; MacKinnon et al., 2018), and still others, to resilient outcomes (advanced cognitive development and stress resistance; Fujioka et al., 2001; DiPietro et al., 2006). We observed that offspring of mothers that were relocated for an average of 9 days over an average of 1.5 relocations during pregnancy were more active, confident, and willing to engage with novelty, and exhibited lower post-stress cortisol during a moderately stressful social separation at 90–120 days of age. Controlling for the genetic and post-natal confounds of prenatal stress introduced when mothers rear their own biological offspring, we compared biologically reared infants with infants cross-fostered to new mothers. Intriguingly although our cross- fostered sample size was small, the notable behavioral consequences were identical across both groups, demonstrating the independence

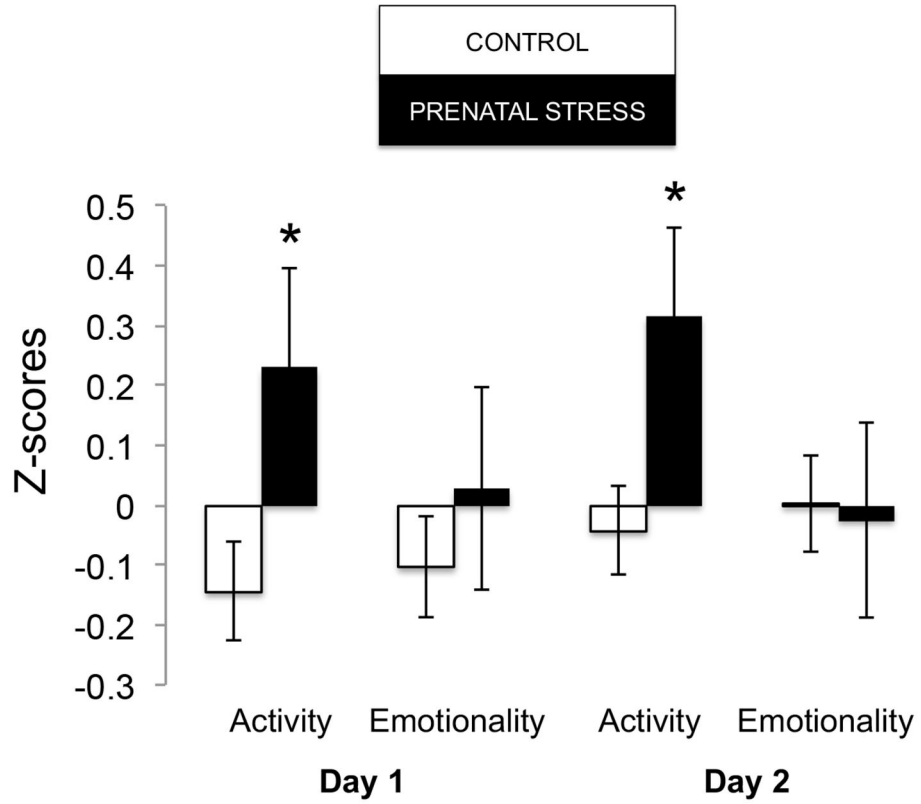

FIGURE 1 | Effects of prenatal stress on Holding cage Activity and Emotionality during biobehavioral assessment between offspring of prenatally stressed mothers ($n = 39$) and control mothers ($n = 149$). Means are presented ± standard error of the mean. $^*p < 0.05$.

of prenatal stress effects from genetic confounds or post-natal maternal factors.

Prior research suggests that prenatal stress is a risk factor for behavioral dysregulation and problematic outcomes in animal and human health (Schneider et al., 1992, 2002; Coe et al., 2003; Gutteling et al., 2005; Ruiz and Avant, 2005; Pryce et al., 2011). In studies conducted on human children, prenatal stress has been linked with both internalizing and externalizing behavior dysregulation (Betts et al., 2014; MacKinnon et al., 2018; Hentges et al., 2019). Moreover, multiple studies have shown a link between prenatal stress and signs of internalizing behavior at an early age. Internalizing behaviors include negative affect, anxiety, depression, and withdrawn behavior (Gutteling et al., 2005; Hentges et al., 2019). For example, in a study measuring the effects of prenatal stress on 6-month-old human infants, prenatal stress and anxiety were found to predict higher levels of negative emotional affect thus increasing the risk for later internalizing behavior (Nolvi et al., 2016). Similarly, Hentges et al. (2019) found that indices of prenatal stress such as perceived stress, anxiety, and depression were indicative of greater child internalizing behavior at ages 3 and 5 years of age. In addition, prenatal stress has also been found to amplify interaction effects amongst a challenging post-natal environment by predicting greater child internalizing behaviors when exposed to greater amounts of prenatal stress (Hartman et al., 2020). Another body of work shows that as children mature, externalizing behavior such as conduct problems and poor impulse control, may emerge (Van den Bergh and Marcoen, 2004; Rice et al., 2010; MacKinnon et al., 2018). However, in the majority of these studies, the link between prenatal stress and externalizing phenotypes is typically not apparent until late childhood or adolescence. Moreover, these studies did not control for post-natal confounds on offspring development. Behaviorally, our monkeys represent a useful model to track these links: in our monkeys, we observed similar patterns of temperamental tradeoffs: externalizing traits (activity, novel object interaction, confidence) were moderately intercorrelated and these traits were inversely associated with internalizing traits (nervousness).

Because the age of our infants (3–4 months) is equivalent to about 1 year of human age, we expected our results to align with younger human children, which largely show signs of internalizing behavior following prenatal stress (Nolvi et al., 2016; Hartman et al., 2020). But prenatal relocation stress did not predict internalizing behavior in our infants. On the contrary, prenatally relocated animals exhibited more active, exploratory, and confident behavior. Upon brief separation from mothers and social groups, within 3 h, prenatally relocated infants engaged in more active exploration of their novel indoor housing, displaying activities such as locomotion, eating, drinking, environmental exploration, and crouching. At the

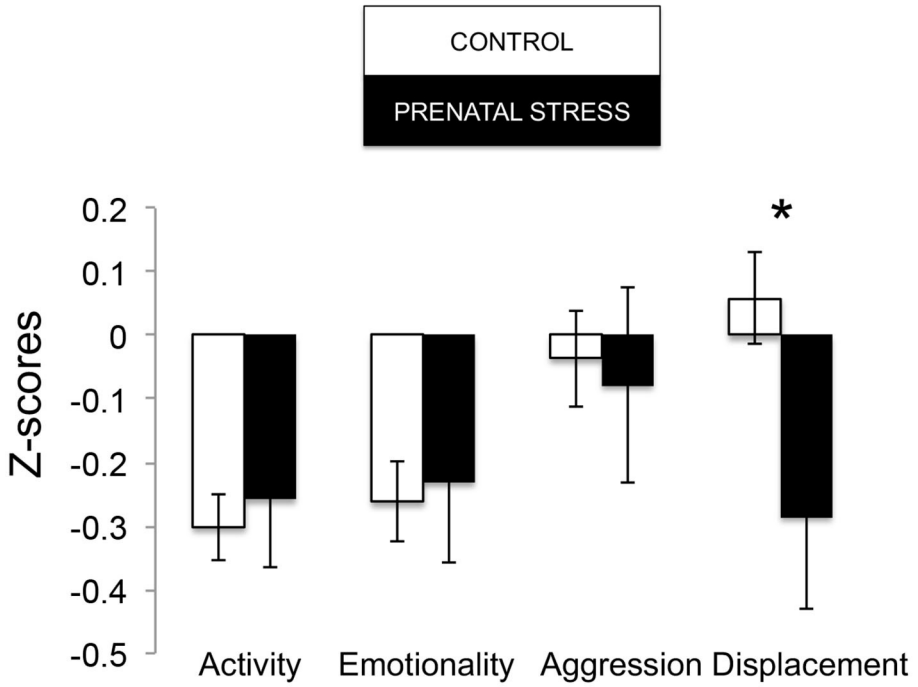

FIGURE 2 | Human Intruder response during biobehavioral assessment between offspring of prenatally stressed mothers ($n = 39$) and control mothers ($n = 149$). Means are presented ± standard error of the mean. *$p < 0.05$.

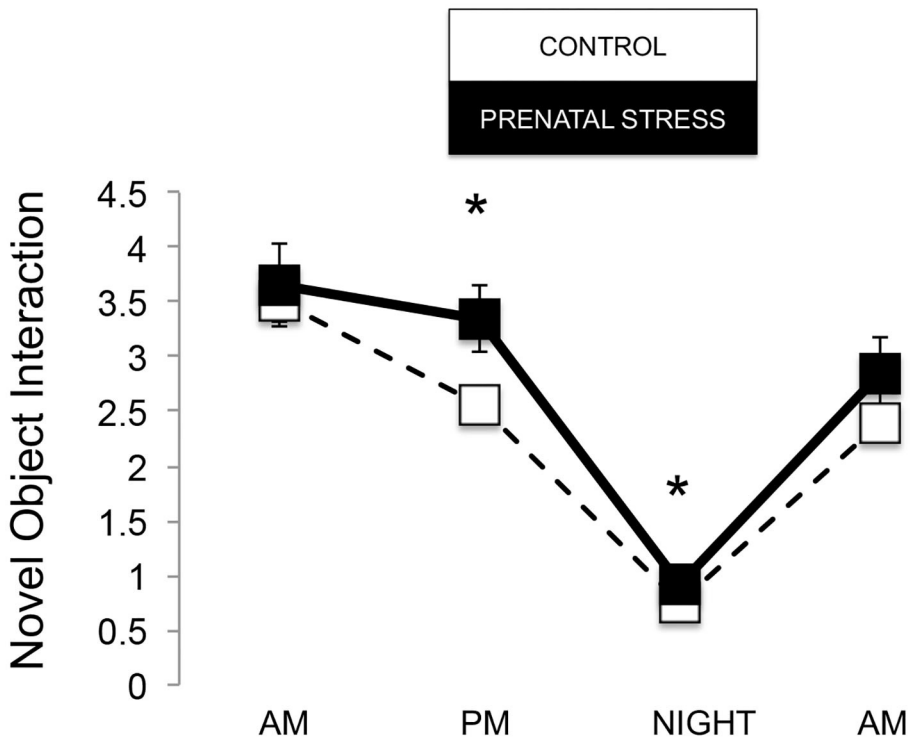

FIGURE 3 | Novel object interaction during biobehavioral assessment between offspring of prenatally stressed mothers ($n = 39$) and control mothers ($n = 149$). Means are presented ± standard error of the mean. *$p < 0.05$.

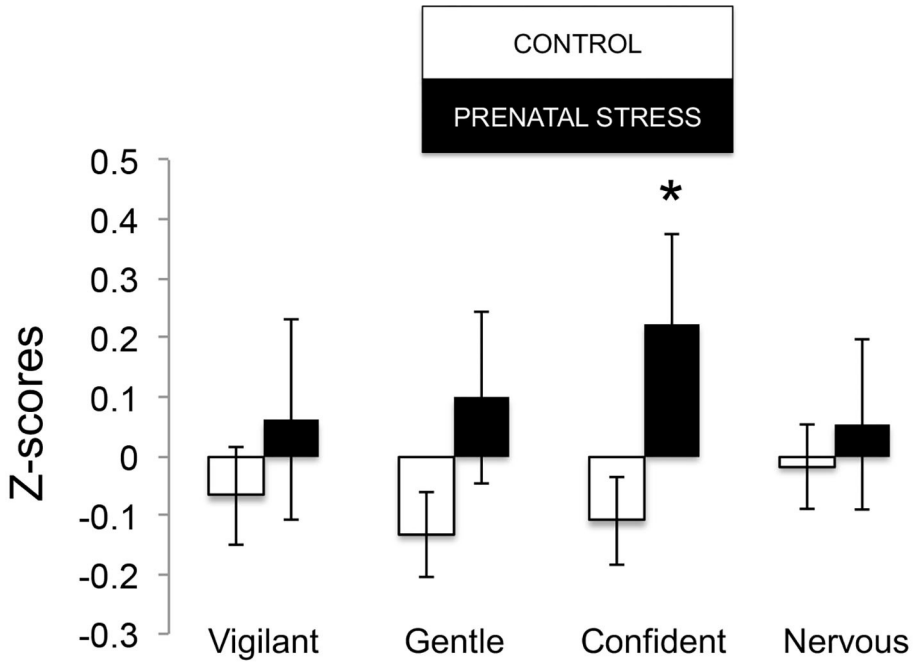

FIGURE 4 | Temperament measures during biobehavioral assessment between offspring of prenatally stressed mothers ($n = 39$) and control mothers ($n = 149$). Means are presented ± standard error of the mean. *$p < 0.05$.

same time during assessment, they did not exhibit differences in emotion-related behavior, which includes cooing, barking, scratching, threatening, and lipsmacking. This pattern continued into the next day, when prenatally relocated infants showed more activity and exploration, but not greater emotionality, which suggests that these infants showed consistent curiosity about, rather than fear of, their temporary environments. Prenatally relocated infants also showed reduced anxiety (displacement behaviors tooth grinding and yawning) in response to a human intruder challenge (a human standing in front of their holding cage at near and far distance, and making eye contact or showing a side profile) compared with Control infants. Human intruder measures were strongly correlated across Profile and Stare conditions and so combined into one measure as we have described previously (Kinnally et al., 2010). Throughout the afternoon and through the night of assessment, prenatally relocated infants interacted more often with novel objects. Finally, at the end of the 25-h assessment, relocated animals were rated as more confident (curious, playful, confident, bold, active) than Control infants. This pattern of behavior suggests an underlying engagement with the environment, and reduced anxiety, during stress. These results, on one hand, may translate to human externalizing behaviors like impulsivity and behavioral disinhibition. Consistent with this idea, we have previously shown that adult macaques that interact with novel objects more quickly (similar to infants in the present study) are more impulsive and aggressive in social contexts, consistent with an externalizing phenotype (Kinnally et al., 2008).

However, we suggest that the pattern of behaviors displayed by prenatally relocated infants may suggest a pattern more consistent with a robust, engaged, and curious response to challenge, rather than an impulsive response. In the face of a novel stressful challenge, these animals adapted well and interacted with their environment positively and flexibly. There is support for the idea that behavioral patterns like these are associated with positive engagement and adaptive stress response in rhesus monkeys. Infant macaques' subjective well-being (rated by trained observers) was positively correlated with greater characteristics of openness, and assertiveness, and negatively correlated with signs of anxiety (Simpson et al., 2019). We have previously shown that while rapid interaction with novelty may suggest impulsivity and adverse social outcomes, sustained and exploratory interest in novelty favors positive social behavior and relationships (Kinnally et al., 2008). In humans, such positive emotionality is associated with greater physical and mental health, such as faster cardiovascular recovery from exposure to stressful conditions (Fredrickson and Levenson, 1998; Lyubomirsky et al., 2005). Interestingly, these personality traits used to distinguish trait- resilient individuals appear to establish in early infancy and maintain their positive correlation with subjective well-being well into adulthood providing signs of early adaptive coping (Tugade et al., 2004). Our results suggest that animals that were relocated while *in utero* may actually be more resilient when later dealing with challenge. This interpretation would be consistent with a prenatal stress inoculation effect, consistent with some human and animal studies (DiPietro et al., 2006; Ehrlich and Rainnie, 2015; Scott et al., 2017; Serpeloni et al., 2019).

Further, our cortisol data suggest that prenatally relocated infants show a lesser cortisol stress response compared with

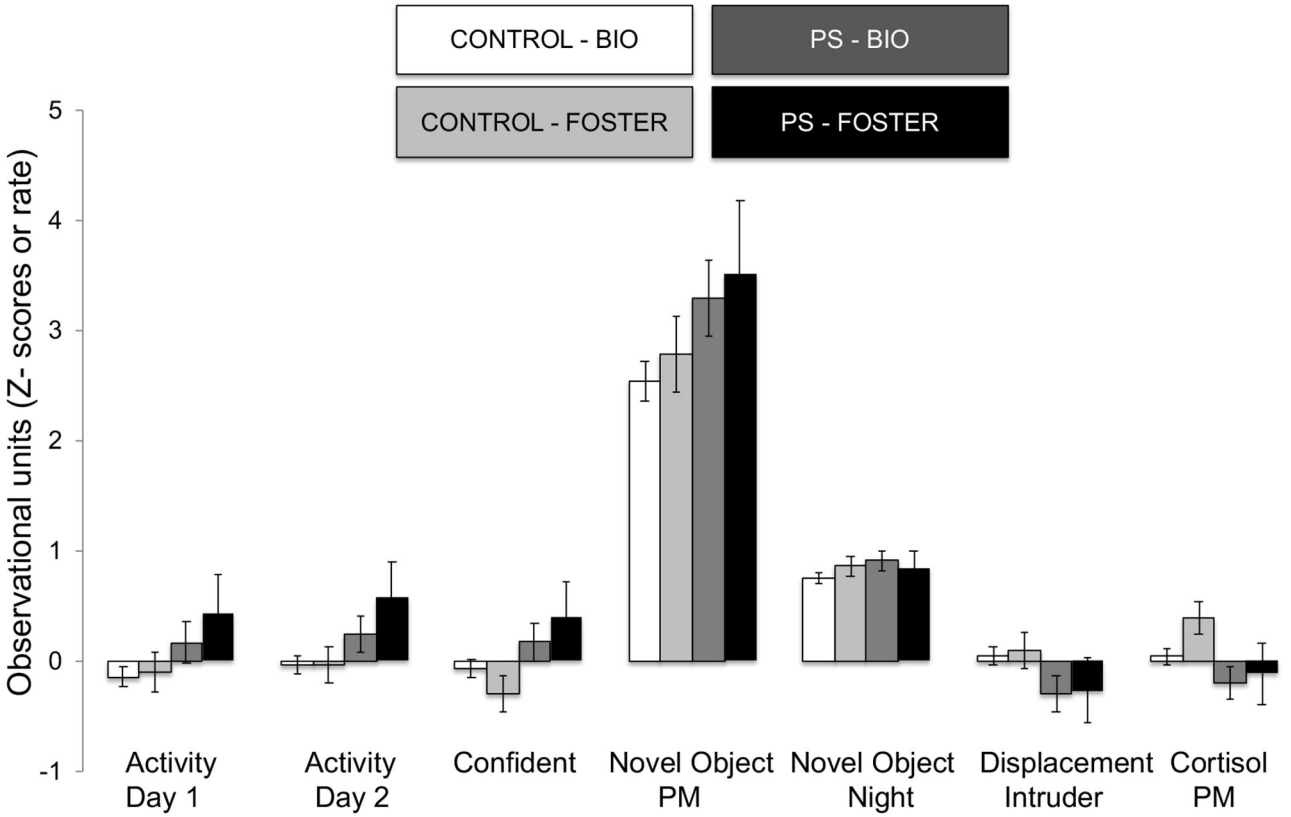

FIGURE 5 | Effects of prenatal stress on significant temperament measures during biobehavioral assessment in biologically reared and cross-fostered infants. Means are presented ± standard error of the mean. CONTROL = No prenatal stress ($n = 149$) PS = Prenatal Stress ($n = 39$), BIO = infants raised by biological mothers ($n = 146$), FOSTER = infants raised by unrelated foster mothers ($n = 42$). $p < 0.05$.

offspring of mothers that were not relocated. Previous studies in macaque prenatal stress have shown the opposite: that prenatal stress increases cortisol output in offspring (Coe et al., 2003), which as we have described, correlated with more anxious behavior. Our combined results point to the opposite effect on our monkeys following prenatal relocation stress, enhancing behavioral and physiological resilience to challenge in infancy.

Our data are consistent with recent syntheses that suggest that prenatal stress can have context dependent influences on individual development (Pluess and Belsky, 2011; Cymerblit-Sabba et al., 2013; Hartman et al., 2018). We suggest that commonplace prenatal stress may confer stress inoculation, while more intense stressors may confer risk (Glover et al., 2010). For example, previous studies utilizing a combined effect of prenatal relocation and stress were arguably somewhat intense—i.e., relocating a female into a darkened room daily for 1 week every 5 weeks, and introducing unpredictable acoustic startle during relocation (Coe et al., 2003). Our temporary relocation may have been relatively mild, as adult females had experienced similar relocations for similar reasons multiple times in their lives. However, because all animals were relocated for reasons that may have added to their stress, like injuries inflicted by other group members, illness, or health problems of their infant, it is possible that relocation was more than a commonplace stressor for our subjects. Our results suggest that our stressor differs from previous experimental stressors in terms of their influence on infants. In Coe et al. (2003), prenatally stressed infants were less engaged with their environment than control infants, while our relocation stress was linked with more engagement with the environment, confidence and reduced anxiety. One reason that our stressor may be considered less intense is because of its ecological relevance: our stressor potentially equates to normative stress naturally encountered throughout macaque (and possibly human) pregnancy. Yet another possibility is that the reason for relocation played a larger role than we were able to detect: the majority of our mothers were relocated due to injuries while a subset of five females were relocated due to illness. Alternatively, the harmful effects caused by the relocation stressor may have also been ameliorated through social buffering when reuniting with friends and family in their home enclosures. Though our preliminary analysis of differences in offspring of females relocated due to illness vs. injury yielded no significance, caution should be taken in interpretation due to our smaller subsample. It is possible that relocation due to injury is a very different experience than relocation due to illness.

Some of the variability in the effects of prenatal stress in human studies may be attributed to correlations with post-natal factors: socioeconomic status (Lobel et al., 1992), a steady support

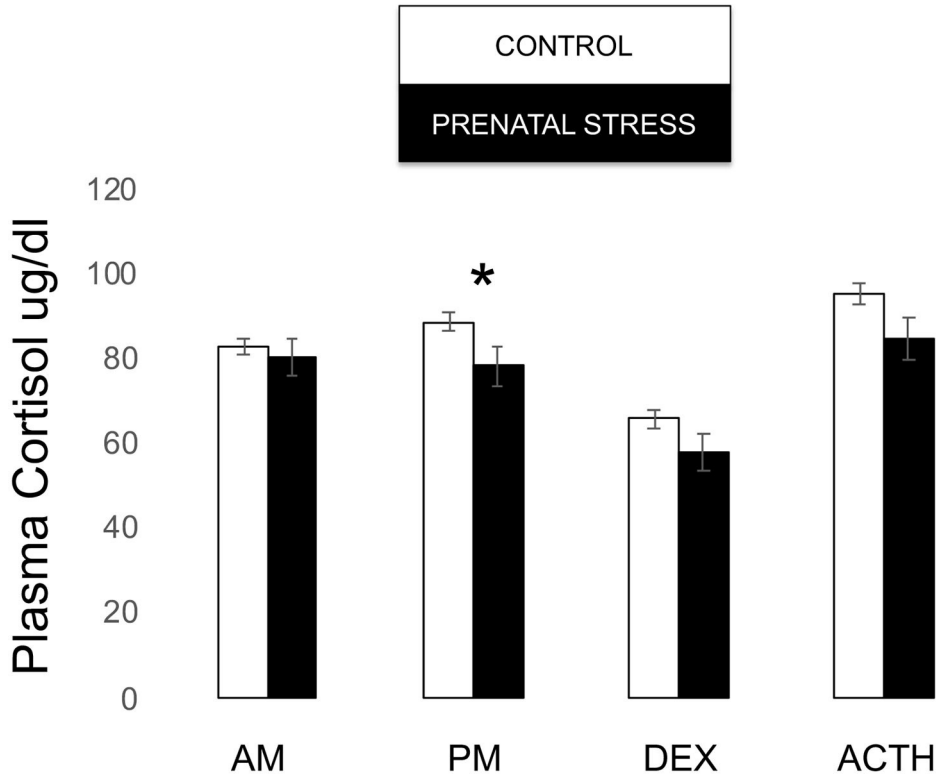

FIGURE 6 | Plasma cortisol measures during biobehavioral assessment between offspring of prenatally stressed mothers ($n = 39$) and control mothers ($n = 149$). Means are presented ± standard error of the mean. *$p < 0.05$.

system (Dunkel-Schetter et al., 1996), or differences in individual maternal attributes (DiPietro et al., 2006) that influence pre- and post-natal development in humans. Translational animal models of prenatal stress are useful to compare the relative effects of genetics, prenatal and post-natal experiences on the developing infant and disentangling nature vs. nurture effects. We compared the effects of our prenatal relocation stressor on infants reared by their biological mothers vs. foster mothers to control for social/environmental influences and ensure that our results were not due to genetic confounds between the mother and offspring (Kinnally et al., 2019). We observed that behavioral effects of prenatal stress were consistent between foster and biologically reared infants, suggesting that prenatal stress had a unique effect on post-natal development independent from interactions with maternal behavior or genetic confounds. This is consistent with a genetically informed human prenatal stress study that compared infants born via *in vitro* fertilization (Rice et al., 2018). Infants born of eggs donors that had experienced prenatal stress but gestational mothers that had not experienced stress showed higher risk for antisocial behavior and anxiety (Rice et al., 2018). Taken together, our results suggest that prenatal stress effects were specific and not mediated by genetic or post-natal correlations with prenatal stress that might arise due to maternal anxiety or chronic environmental stress in the social group.

Maternal factors are to be considered an important direct contributor to an infant's indirect prenatal and direct post-natal environment. Though post-natal factors did not appear to confound our prenatal stress effects, as we might expect, some maternal characteristics influenced infant biobehavioral development. Features of the social environment and maternal care played a role in infant biobehavioral development. In our study, lower maternal rank predicted higher activity and emotionality on Day 1 with continued emotionality into Day 2. Offspring of lower ranked mothers were also rated as more vigilant. This is consistent with the notion that the social environment of lower ranked mothers heightened awareness and emotionality in infant offspring early in life, wither due to the infant's direct experience in the social group or indirect effects through the mother's behavior or physiology. We also observed a global effect of SPF status on biobehavioral development. SPF enclosures are defined by at least two features: they were founded with so-called nursery reared (NR) founders to breed out zoonotic diseases, and they house monkeys free of these specific pathogens. NR entails separation from mothers at birth and rearing in a socially restricted environment. In adolescence, infants are moved to large SPF social groups. In the present study, our SPF infants showed more emotionality, less interaction with novel objects and more intense reactions to a human intruder. The results are consistent with a more anxious phenotype. We have previously shown that paternal line descendants of NR males show increased levels of anxiety (Kinnally et al., 2018), so the fact that SPF animals likely have

more NR ancestors may explain this effect. While there were no global effects of maternal behavior in the omnibus model, *post-hoc* tests showed that neutral mothering predicted more activity on Day 1, higher ratings of temperamental gentleness and less temperamental nervousness. Neutrality in maternal care is used to describe dyadic interactions in which the infant initiates a proactive interaction toward the mother and the mother maintains proximity but does not respond in any particular proactive manner (Kinnally et al., 2018). Each of these maternal factors were controlled for in the overall analysis, and were independent of prenatal stress effects. These results highlight the importance of complex post-natal maternal factors on infant development, in addition to and independent of commonplace prenatal stress.

A major limitation of this study is that we cannot infer direct causality on the effects of prenatal stress because we did not specifically manipulate relocation stress. Since our prenatal relocation data were retrospective and opportunistic, we were not able to control for potential mediating factors related to aspects of relocation, such as reason for relocation or length of stay. For example, most females that were relocated were relocated due to injuries incurred in the social group, and relatively fewer due to illness. We did control for these variables in our early analyses and did not find a significant correlation between reason for relocation or length of relocation stay and our outcomes. However, it is possible that behaviors that increase risk for injury in social groups may be correlated with other life stressors. We also cannot entirely preclude that correlational factors associated with relocation may have contributed to our effects. For example, we examined whether the number of ketamine exposures, which was higher in prenatally relocated females, explained the effects of prenatal relocation, but the number of prenatal ketamine exposures alone did not drive our behavioral effects. But because all prenatally relocated females were treated with ketamine (as were most control females, during regularly scheduled health checks) during pregnancy, we cannot preclude an interaction effect between ketamine and relocation. Some studies have reported an effect of neuronal loss, cognitive deficits, and depressive-like offspring behavior as a result of maternal exposure to ketamine during prenatal stages (Zhao et al., 2014). Although further exploration is needed to investigate neural effects of ketamine in our subjects, our results did not align with the behavioral irregularities observed in these studies. Moreover, the likelihood that ketamine specifically confounds our prenatal stress condition seems remote, as additional studies have linked ketamine (and combined genetic factors) with biobehavioral outcomes suggesting poorer attention and internalizing behavior (Capitanio et al., 2012), while we believe our results point to resilient outcomes arising from prenatal manipulation. However, ketamine exposure has been linked with antidepressant effects in the exposed individual (Berman et al., 2000), so it remains possible that that prenatal ketamine played a role in the resilient outcomes we observed. This notion is somewhat undermined by our finding that neither the reason for relocation, nor number of ketamine exposures were statistically linked with our outcomes, this will be an important experimental control in future studies. Another possibility is that prenatal stress shifted aspects of development that we did not capture that impacted our results. For example, if prenatal stress led to premature birth, this could have affected our results. We do not believe this was the case, however, as our prenatal stress group did not differ in birth weight from controls. Our results however suggest that experimental manipulation of commonplace stress during pregnancy may yield a useful monkey model of enhancing stress resilience during infant neurodevelopment.

In conclusion, our findings support the notion that stress generated through maternal relocation during pregnancy led to a more active, confident and engaged phenotype in infant rhesus monkeys. One interpretation is that this commonplace prenatal stressor conferred resilience to prenatal stress. These findings highlight a need for future work (particularly experimental work) to address how mild prenatal stress may optimize behavioral stress response. Identifying these thresholds could help pave the way in exploring intervention options in cases of highly adverse behavioral outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by UC Davis Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LC: conceived of and executed study and wrote paper. JC: collected biobehavioral data, edited paper, and funded research. EK: senior author, statistical analyses, edited paper, and funded research. All authors contributed to the article 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/fnbeh.2021.641795/full#supplementary-material

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REVIEW
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White-Matter Repair as a Novel Therapeutic Target for Early Adversity

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Early adversity (**EA**) impairs myelin development in a manner that persists later in life across diverse mammalian species including humans, non-human primates, and rodents. These observations, coupled with the highly conserved nature of myelin development suggest that animal models can provide important insights into the molecular mechanisms by which EA impairs myelin development later in life and the impact of these changes on network connectivity, cognition, and behavior. However, this area of translational research has received relatively little attention and no comprehensive review is currently available to address these issues. This is particularly important given some recent mechanistic studies in rodents and the availability of new agents to increase myelination. The goals of this review are to highlight the need for additional pre-clinical work in this area and to provide specific examples that demonstrate the potential of this work to generate novel therapeutic interventions that are highly needed.

Keywords: early adversity, early life stress, myelin, white matter, animal models, translational research, rodents, non-human primates

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INTRODUCTION

EA in the form of childhood neglect, poverty, and maltreatment is now recognized as one of leading causes for chronic mental and medical conditions in adulthood (Anda et al., 2006; Nemeroff, 2016; Teicher and Samson, 2016; White and Kaffman, 2019). Roughly half of all childhood psychiatric conditions are linked to EA (Green et al., 2010) and the cumulative economic burden of EA is estimated at $2 trillion in the United States alone (Peterson et al., 2018). Interventions that are able to correct maladaptive behaviors and cognitive deficits associated with EA are highly needed and will have tremendous clinical and economic impact. In this review we focus on the role that abnormal myelin development plays in mediating some of the psychiatric consequences of EA and propose that adaptive myelination may provide an important and novel therapeutic target for normalizing cognitive and behavioral deficits associated with EA.

Myelin is a vertebrate-unique evolutionary feature in which specialized glia cells in the central nervous system, known as oligodendrocytes, wrap myelin sheaths along axonal segments. This process starts at around birth and matures in early adulthood (Foster et al., 2019). Recent work from several groups has highlighted important new features of myelin development and its role in adult plasticity (Chorghay et al., 2018; Foster et al., 2019; Liu et al., 2020). During development, myelin facilitates the selection of functional connections and promotes circuit synchronization and

oscillatory outputs (Pajevic et al., 2014; Chorghay et al., 2018; Foster et al., 2019; Liu et al., 2020). This process is tightly linked to neuronal activation and is sensitive to environmental changes early in life (Foster et al., 2019; Liu et al., 2020). Not surprisingly, exposure to EA in the form of neglect, deprivation and maltreatment alters white-matter development in humans, non-human primates and rodents (reviewed in **Table 1**). Although some progress has been made in identifying the mechanisms by which EA alters myelin development, the findings are inconsistent and many key questions have not been addressed. Theses inconsistencies are likely due to the heterogeneity in the type, timing, and complexity of the adversity, as well as genetic vulnerability and brain region-specific changes in activity-dependent myelination (aka ***adaptive myelination***).

The central assertion of this review is that animal models can untangle many of these challenges and clarify how different types of adversities impair myelination in a manner that persists into adulthood and impacts cognition and behavior. Further, we note that this area of translational research has received relatively little attention and call for additional studies. In support of this assertion we note that myelin development is highly conserved across diverse mammalian species (Bergles and Richardson, 2015). Key variables of adversity can be rigorously controlled in animals and sophisticated tools are available to manipulate neurons and oligodendrocytes in animals. Further, the availability of human imaging techniques to assess myelination in animals allows for more direct cross-species comparisons (Kaffman et al., 2019), and recent progress in the development of therapeutic interventions that enhance adaptive myelination (Murphy and Franklin, 2017) provides a novel strategy to reverse myelin and behavioral abnormalities seen in animals exposed to EA.

We start with a general review of myelin function in nerve conduction, network synchronization, behavior, and summarize the role that oligodendrocytes play in network selection during development. Next, we review a large body of work showing that EA alters myelin development in humans, non-human primates and rodents, highlighting important mechanisms that appear to guide these changes in rodents. We close by outlining key unresolved questions and the work needed to address these issues.

MYELIN MODULATES NETWORK PROPERTIES IN RESPONSE TO ENVIRONMENTAL CUES

Oligodendrocytes (**OL**) are specialized cells that wrap lipid-rich myelin sheaths, known as ***internodes***, around large-diameter axons. Internodes with different lengths are spaced along axons at variable densities and are interspaced by specialized myelin-free segments enriched with ion channels known as ***nodes of Ranvier*** (**Figure 1**) and reviewed in Chorghay et al. (2018), Foster et al. (2019). Internodes prevent current leakage along large axonal segments leading to saltatory conduction with increased conduction velocity and greater reliability in signal transduction (Mitew et al., 2014; Chorghay et al., 2018). Conduction velocity is modulated by myelin thickness and internode length and density along the axon. These properties are highly regulated by axonal activity and environmental cues, a process known as ***adaptive myelination*** (Gibson et al., 2014; Foster et al., 2019; Suminaite et al., 2019). Adaptive myelination promotes network synchronization, the selection of functional circuits during development, improved learning, and behavioral modifications later in life (Pajevic et al., 2014; Kaller et al., 2017; Chorghay et al., 2018). See Sections "Myelin and Behavior," "Mechanisms of Myelin Development," and "EA Causes White-Matter Abnormalities in Humans, Non-human Primates and Rodents" below for more details.

Myelin also plays an important role in addressing energetic demands of neuronal networks (Saab et al., 2013; Philips and Rothstein, 2017). For example, insulation of large axonal segments minimize current leakage and reduces energetic costs associated with neuronal impulse propagation (Mitew et al., 2014). Neuros have small reserves of glycogen storage and therefore rely on metabolic support provided by astrocytes and oligodendrocytes, with oligodendrocytes playing a particularly important role in transporting lactate into adjacent axons Saab et al. (2013), Philips and Rothstein (2017). Monocarboxylate transporters such as MCT1 located in ***uncompacted myelin*** allows for the transport of lactate into the adjacent axon where it is converted to ATP (**Figure 1**). This metabolic coupling is highly regulated by axonal activity and is essential for its function, for reviews see Saab et al. (2013); Philips and Rothstein (2017). The importance of this "symbiotic" relationship is highlighted by work showing that oligodendrocyte-specific deletion of MCT1 causes axonal degeneration in aged mice (Lee et al., 2012; Philips et al., 2021). Perturbation in components of ***compacted myelin***, such as myelin basic protein, which are responsible increasing conduction velocity do not cause axonal degeneration, indicating that compacted and uncompacted myelin have different roles. This distinction may explain why some myelin changes associated with EA are more difficult to reverse later in life and why apparently similar perturbation in myelin structure may cause different functional consequences (Bick et al., 2015; Philips and Rothstein, 2017; McCarthy-Jones et al., 2018; De Leon Reyes et al., 2020).

Myelin also inhibits neuronal plasticity. This is mediated by the expression of ligands such as Nogo-A and MAG by oligodendrocytes (**Figure 1**, red no entry signs). These ligands restrict axonal sprouting and prevent axonal regeneration after spinal cord injury, stroke, and traumatic brain injury (Geoffroy and Zheng, 2014). Increased levels of myelination in the visual cortex during the juvenile period is responsible for closing the critical period in response to monocular deprivation (McGee et al., 2005). The ability of myelin to promote plasticity in activated axons while inhibiting sprouting in neighboring axons is a unique feature that may promote the selection of functional circuits during development and later in life.

In summary, myelin increases processing speed and accuracy at significantly reduced energetic costs. Myelin also provides metabolic support, helps synchronize network oscillation and restricts axonal sprouting. These properties are influenced by environmental cues and are necessary for selecting functional circuits and to improve learning and perception throughout life.

TABLE 1 | EA impairs myelin development in humans, non-human primates and rodents.

References	Experimental design	Summary of findings
Humans		
Teicher and Samson, 2016	Systematic review	Reduction in the size of the corpus callosum and reduced fractional anisotropy in the corpus callosum as the most robust findings in humans exposed to EA. Also, summarizes findings in other white matter tracks including the inferior longitudinal fasciculus, superior longitudinal fasciculus/arcuate fasciculus, uncinated fasciculus, cingulum bundle and the fornix
McCarthy-Jones et al., 2018	Diffusion MRIs were used to assessed the effects of EA on microstructural integrity (i.e., fractional anisotropy, tissue fractional anisotropy and free water diffusion) in 7 white tracks that have previously been shown to be altered by childhood adversity. EA was assessed using Childhood adversity questionnaire (no childhood adversity n = 34, low score of EA n = 58, high score of EA n = 55, ages 37–42). ROI included cingulum bundle, corona radiata, corpus callosum, superior longitudinal fasciculus, inferior frontal-occipital fasciculus, fornix and the uncinate fasciculus.	Found significant reduction in FA in 6 out of the 7 ROI when using EA as a categorical variable, with the fornix being the only ROI that was not significant using categorical analysis. When EA was used as a continuous variable all 7 ROI showed significant changes in FA using regression analysis.
Bick et al., 2015	Randomized clinical trial examining the effects of adoption into foster families on white matter development in children that were institutionalized in a Romanian orphanage during the first 2 years of life and then either stayed in the institution (IG) or adopted (AG). Institutionalized group (IG) n = 26, adopted group (AG) n = 23, never institutionalized group (CTL) n = 20, ages 8–11 when imaged.	Normalization of FA were seen in the AG but not the IG groups in several white tracks including the left external capsule, right external capsule, retro-lenticular internal capsule, right cingulum, right anterior corona radiata, left superior corona radiata, and the medial lemniscus. No remediation of foster care were seen for the corpus callosum and the superior corona radiatum.
Tanti et al., 2018	Postmortem assessment of markers of myelination in the ventro-medial prefrontal white matter region in individuals that died by suicide in the context of depression and EA (DS-EA), depression but without EA (DS) and no depression and no EA, CTL (n = 18 males per group, average age 37)	Reduced density of Olig2 (a general marker of OL) and increased density of Nogo-A, APC, Mash1 (markers of mature OL) in DS-EA compared to DS and CTL groups with some evidence that these effects were more pronounced in younger individuals. DS-EA and DS groups show reduced MBP levels but there was no effect of EA on any markers of myelination (e.g., PLP, CNP, MAG, MOG, MOBP). The authors propose that EA causes precocious maturation of OPC in the prefrontal cortex.
Non-human primates		
Sanchez et al., 1998	MRI changes were assessed in rhesus monkeys (*Macaca mulatta*) exposed to nursery (singly caged, 2–6 months or 2–12 months) or semi-naturally social rearing, from 2 to 12 months, with behavioral testing done at 12M and MRI at 18M (Nursery, n = 9 males, Control, n = 11 males)	Single caging caused a reduction in corpus callosum size, mainly in posterior/caudal regions. Reduced corpus callosum size persisted after 6 months of peer rearing and was correlated with slower acquisition, but not performance, in the delayed non-matching to sample task. Reduced white matter volumes were also seen in the prefrontal cortex and the parietal cortex.
Coe et al., 2002	Pregnant female rhesus monkeys (*Macaca mulatta*) were exposed to daily 10 min acoustic startle during the third trimester (gestational ages 90–140). After birth, infants were raised in nursery for 1 month and then in a small peer-group. MRIs were done at 7–11 months of age. CTL n = 5, Prenatal stress n = 11, roughly 50% males.	There was a significant interaction between prenatal stress and sex for corpus callosum size with reduced volumes in males and increased volume in females. These changes were most pronounced in posterior/caudal regions of the corpus callosum.
Jackowski et al., 2011	Mother-infant dyads of Bonnet monkeys (Macaca radiata) were exposed to variable foraging demands (VFD) when infants were 2–6 months. In adulthood male monkeys were scanned with MRI (CTL n = 9, VFD n = 14, roughly 5 year old. Two years later some of the animals were tested in the intruder test and processed for postmortem assessment of corpus callosum mid-sagittal surface area.	Exposure to VFD early in life was associated with reduced corpus callosum size using a region of interest, but not whole brain voxel volumetric assessment. Reduced corpus callosum was highly correlated with increased emotional reactivity in the intruder test and was confirmed using postmortem analysis performed 2 year after the initial MRI study. Together these findings indicate that exposure to VFD causes long-term reduction in corpus callosum size.
Howell et al., 2013	A naturalistic childhood maltreatment model in rhesus monkeys (*Macaca mulatta*) was used to assess the effects of childhood maltreatment on white matter organization (FA, RD, AD) using dMRI in adolescent animals. CTL n = 10 (6F/4M), MT n = 9 (5F/4M), ages around 4 years.	Maternal maltreatment was associated with reduced FA in the corpus callosum and several other white- matter tracks including the occipital white matter and brain stem. Reduction in FA was correlated with elevated corticosterone levels at 1 month of age when maltreatment was at its peak. Reduced FA in occipital WM was correlated with increased aggression in adolescence.
Rodents		
Makinodan et al., 2012	Tested the effects of social isolation during the juvenile period (P21-55) on social behavior, PFC function (non-match to place task) and myelination in mice.	Social deprivation during a sensitive period of development (P21-P35) causes abnormal maturation of oligodendrocytes in the PFC. Abnormal myelination in the PFC is due to impaired Erbb3 receptor activation in oligodendrocytes leading to social and PFC deficits that are not easily reversed later in life.
Yang et al., 2017	Sprague Dawley rats were exposed to a split-litter maternal separation model, in which half of the pups were isolated from littermates and dam for 3 h from P3-21. The remaining pups were handled but immediately returned to the home-cage and served as controls. Pups were weaned at P21 and assessed for myelination at P21 or as young adults (P60). The exact timing for the behavioral work was not specified, except for those rats tested after administering the WNT antagonist XAV939.	MS impaired myelin formation in the PFC, but not in other brain regions such as the corpus callosum. Deficits in myelination were seen in P21 pups and persisted into adulthood. The authors provide compelling evidence that increased WNT signaling prevents the maturation of oligodendrocytes in the PFC of rats exposed to MS, including data showing that blocking WNT signaling can rescue behavioral and myelination deficits seen in MS rats. They further show that demyelinating injury to the PFC causes similar behavioral abnormalities to those seen in rats exposed to MS.

(Continued)

TABLE 1 | Continued

References	Experimental design	Summary of findings
Teissier et al., 2020	Balb/cj mouse pups were exposed to 3 h of MS from P2-14. Effects of MS and neuronal activation/inactivation on myelination were tested at P15 and adulthood (P80) in the PFC. Effects on behavior were tested in adulthood.	MS caused reduced expression of immediate early activation genes in the PFC (i.e., reduced neuronal activation) that was associated with accelerated maturation of OPC into mature OL at P15, and reduced OPC density, but not mature OL in the PFC of adult mice. Using chemogenetic manipulation of neurons in the PFC the authors showed that inactivation of PFC neurons from P2-14 in mice raised under CTL condition is sufficient to recapitulate the myelination and behavioral deficits seen in MS mice. Further, activation of neurons in the PFC during the first two weeks of life eliminated the behavioral abnormalities seen in MS mice.
Berrebi et al., 1988	Rat pups were separated from their dam and littermates for 3 min from P1-20 (i.e., handled) and corpus callosum size was assessed at P110 and P225.	Handling increased corpus callosum size in P110 (fully adult rats) males and reduced it in females. The interaction between sex and rearing for corpus callosum size was no longer significant at P215 (middle-age rats).
Duque et al., 2012	Used maternal separation with early weaning (MSEW) in which C57BL/6j pups were exposed to prolonged maternal separation (4–8 h) from P2-P17 and weaned at P17. Control (CTL) pups were left undisturbed and weaned at P23. Stereological and DTI measurements were done in adult male mice (P75-95).	MSEW adult mice showed reduced total brain volumes that was most pronounced in the left hemisphere and was particularly notable in the cortex and the hippocampus. Reduced gray matter volume were associated with abnormal myelination in several white matter tracks including reduced myelination and FA in the rostral and medial regions of the corpus callosum. Again, these were more pronounced in the left hemisphere and were associated with increased Olig2 density in the left CC and PFC of MSEW male mice.
White et al., 2020	Balbc/Byj mice pups were exposed to unpredictable postnatal stress (UPS). UPS includes raising pups with limited bedding and nesting from P0-25 and exposing them to unpredictable 1 h maternal separation/nest disruption on P14, P16, P17, P21, P23, P25. In adulthood (P75-90) mice were processed for high resolution dMRI (6 mice per rearing condition and sex, for a total of 24 mice)	Whole brain voxel analysis found reduced volume and FA in the rostral regions of the corpus callosum in UPS mice (cluster size > 25, FDR < 0.1). These changes were seen in both hemispheres with similar outcomes in males and females. UPS mice also showed reduced global network efficiency and increased in small-woldness.

AD, axial diffusivity; AG, adopted group; dMRI, diffusion MRI; EA, Early adversity; FA, Fractional anisotropy; FDR, false discovery rate; IG, institutionalized group; MS, Maternal separation; MSEW, maternal separation with early weaning; OL, Oligodendrocytes; OPC, Oligodendrocytes progenitor cells; P21, postnatal day 21; PFC, prefrontal cortex; RD, radial diffusivity; ROI, region of interest; UPS, unpredictable postnatal stress; VFD, variable foraging demands.

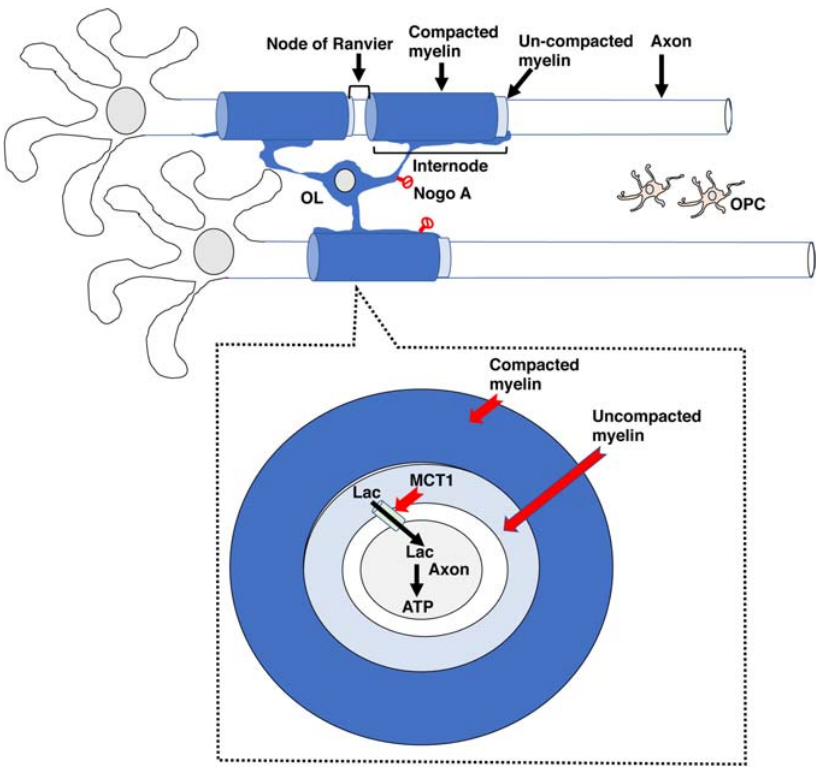

FIGURE 1 | The basic myelin components. A single oligodendrocyte can myelinate several neighboring axons by forming internodal myelin sheaths that are interspaced with myelin free zones known as nodes of Ranvier. Compacted myelin (dark blue) increases nerve conduction and prevents axonal sprouting by expressing ligands such as Nogo A (no entry red signs), whereas uncompacted myelin (light blue) provides metabolic support to underlying axonal segment by expressing channels such as MCT1. Abbreviations: OL- mature oligodendrocyte, OPC- oligodendrocyte progenitor cell.

MYELIN AND BEHAVIOR

Rapid changes in myelination are seen in response to different forms of learning, social cues, and stress, with a growing body of work suggesting that this form of myelin plasticity plays an important role in modifying circuit output, cognition, and behavior (Xiao et al., 2016; Kaller et al., 2017; Chorghay et al., 2018; Liu et al., 2020). For example, elegant work by McKenzie et al. showed that training mice to run on a wheel with missing rungs (aka complex running wheel) induces the differentiation of OPC into mature OL in the corpus callosum and that blocking this process impairs the acquisition but has no effect on the retention of this task after it was acquired (McKenzie et al., 2014). Increase in OPC differentiation is seen within 2.5 h of exposure to complex wheel running and this rapid response was required for maximize acquisition of this skill within this time frame (Xiao et al., 2016). Together these findings demonstrate rapid OPC differentiation and *de novo* myelination improve performance in complex procedural tasks.

Steadman et al. have extended these findings to show that *de novo* myelination is necessary for the consolidation/retrieval of spatial memory in the Morris water maze and contextual fear conditioning (Steadman et al., 2020). Consolidation of spatial memory was associated with increased synchronization between the hippocampus and the anterior cingulate of the prefrontal cortex and this enhanced synchronization was blocked when *de novo* myelination was prevented. Together, these findings establish an important link between *de novo* myelination, network synchronization, and spatial learning (Steadman et al., 2020).

Prolong social isolation reduced myelination in the PFC and was associated with a decreased social interaction in adult mice (Liu et al., 2012). Interestingly, administration of the anti-muscarinic agent Clemastine fumarate increased myelination in the PFC and normalized social avoidance in socially isolated mice (Liu et al., 2016). These observations suggest that social interaction is necessary for maintaining adequate levels of *de novo* myelination in the PFC and demonstrate the therapeutic utility of agents that increase *de novo* myelination for restoring normal social behavior (**Figure 2**).

Pan et al. (2020) showed that contextual fear conditioning was associated with a time- dependent increase in *de novo* myelination in the PFC. Increased myelination was seen roughly 14 days after fear learning and was not seen in the hippocampus or the amygdala. This time-dependent myelination was required for remote (i.e., 28-day after training) but not acute learning (i.e., 24 h post-training). Calcium imaging showed significant changes in neuronal activation in response to remote learning that required *de novo* myelination. Administration of Clemastine fumarate increased remote fear learning in a manner that required *de novo* myelination (Pan et al., 2020), further demonstrating the ability of these agents to modify complex behavior in adulthood.

In summary, *de novo* myelination plays a critical role in different forms of learning including procedural, spatial, and contextual fear conditioning. Myelin-dependent changes in behavior are linked to changes in synchronization and reorganization of neuronal network in specific brain regions, most notably the PFC. Importantly, agents that increase *de novo* myelination promote learning and can be used to compensate for behavioral deficits associated with environmental deprivation (**Figure 2**).

MECHANISMS OF MYELIN DEVELOPMENT

Myelination during development is a protracted and highly coordinated process, starting at around birth and continuing into early adulthood. During development, myelination progresses

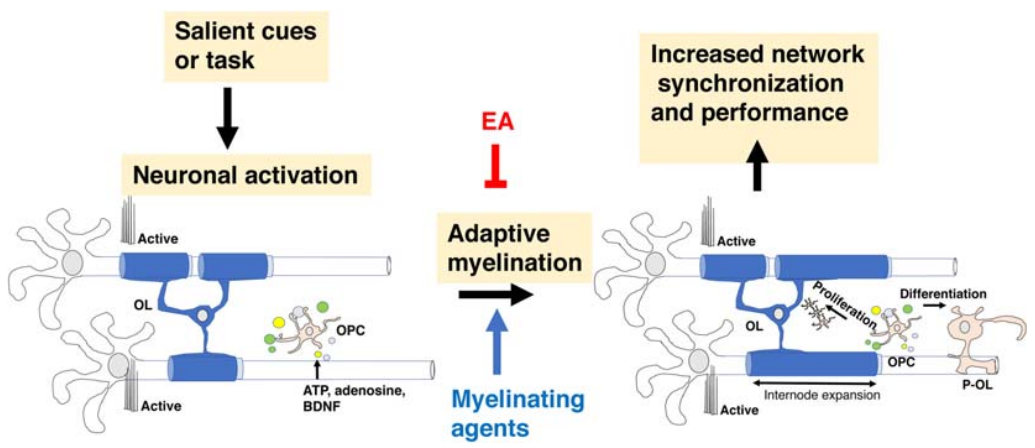

FIGURE 2 | Adaptive myelination in adulthood. Environmental cues increase neuronal activity in specific circuits, leading to the release of ligands such as glutamate, adenosine and BDNF (small colored circles) that promote the expansion of existing internodes. In adult rodents, these ligands drive OPC proliferation and differentiation but this aspect of *de novo* myelination seems negligible in healthy adult humans. Adaptive myelination in adulthood increases network synchronization and improves performance in a variety of tasks. Myelinating agents such as Clemastine fumarate increase adaptive myelination while some forms of EA or prolonged social deprivation in adulthood impair it. EA, early adversity; OL, mature oligodendrocyte; OPC, oligodendrocyte progenitor cell.

in a caudal to rostral fashion that corresponds with maturation of sensory-motor, language, cognition, and executive functions respectively, **Figure 3A** (Mitew et al., 2014; Kaller et al., 2017; De Leon Reyes et al., 2020). Although adaptive myelination in adulthood and developmental myelination share some similarities, important differences exist that are particularly relevant to questions about long-term impact reversibility. Here we summarize key features of myelin development during the postnatal period and highlight their relevance to abnormalities seen in different forms of EA and for therapeutic interventions.

High degree of redundancy ensures robust myelin development under diverse environmental conditions or genetic perturbations (Mitew et al., 2014; Bechler et al., 2018). This redundancy has made it difficult to identify specific pathways that are indispensable for myelin development in the CNS (Mitew et al., 2014; Bechler et al., 2018) and suggest that the effects of EA on myelin development are likely to be multi-factorial and perhaps not simple to correct.

Multipotent neural stem cells differentiate into self-replicating oligodendrocyte progenitor cells (**OPC**), a process regulated by soluble factors such as sonic hedgehog, WNT/β-catenin, and bone morphogenic proteins (Ortega et al., 2013; El Waly et al., 2014; Mitew et al., 2014; Sanchez and Armstrong, 2018). Three waves of OPC migrate and populate the developing brain (Kessaris et al., 2006; Bergles and Richardson, 2015). The first wave is composed of Nkx2.1 positive cells that originates from the ventral neuroepithelium and populates the telencephalon by E16.5. A second wave of Gsh2-positive OPC originates from neighboring sections of the ventral neuroepithelium and arrives to the forebrain soon after the first wave. These embryonic waves are followed by a distinct third wave of Emx2-positive cells. This third wave is generated after birth within the cortex and populates only dorsal regions of the telencephalon such as the cortex and the corpus callosum. In the following 10 days after birth, the Nkx2.1 cell population is eliminated while the number of Emx2-positive cells in the dorsal telencephalon increases to levels that are similar to those of Gsh2-positive OPC. These changes are driven by the production of large numbers of Emx2 and Gsh2, but not Nkx2.1 OPC from the subventricular zone during the first two weeks of life, a process that continues albeit at a lower rate later in life, **Figure 3B** (Ortega et al., 2013; El Waly et al., 2014; Sanchez and Armstrong, 2018). Ablating Gsh-2 OPC leads to expansion

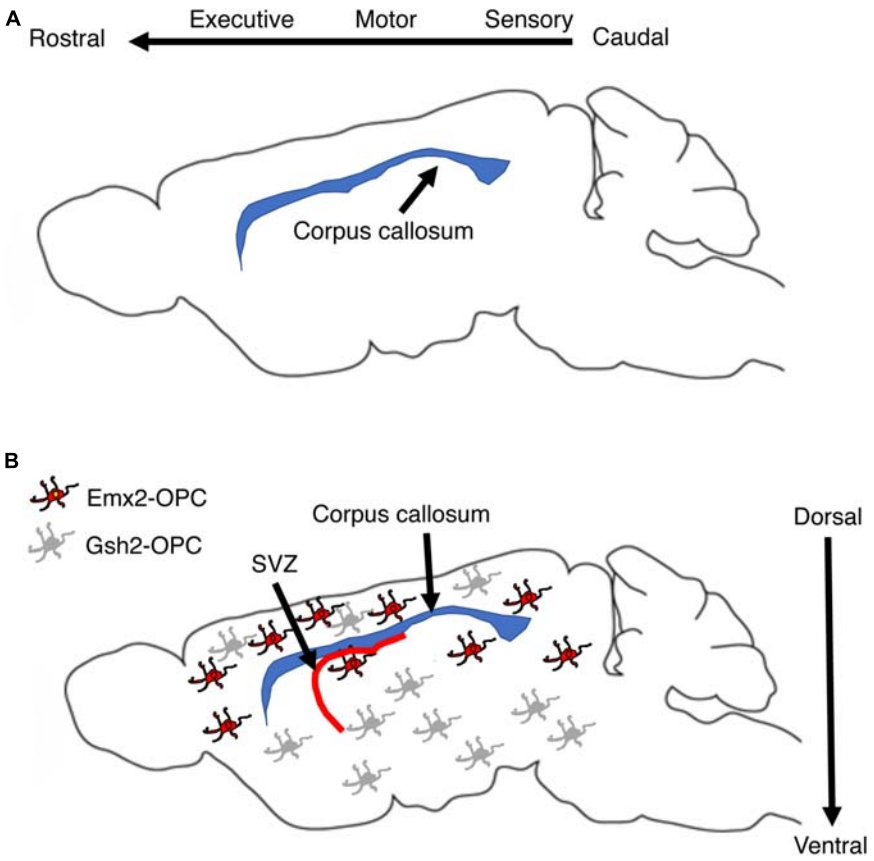

FIGURE 3 | Corpus callosum and myelin development. **(A)** Myelination in the corpus callosum proceeds from caudal to rostral to ensure sequential maturation of circuits that enhance attachment and escape behavior early in life. **(B)** OPCs are a large, heterogenous and somewhat redundant cell population that undergoes significant changes in composition during the first 10 days after birth. Emx2-OPC (red) populate the dorsal telencephalon, appear to be mammalian specific, and are highly responsive to demyelinating conditions. Gsh2-OPC (gray) are present in both the dorsal and ventral telencephalon. Both Emx-2 and Gsh2 OPC continue to be generated throughout life from the SVZ (red). OPCs, oligodendrocyte progenitor cells.

of Emx2 population and vice versa, suggesting that heterogenous and somewhat redundant population of OPC competes for space in the developing telencephalon (Richardson et al., 2006). The observations that Emx2-positive OPC are restricted to dorsal areas and that equivalent wave of dorsally generated cortical OPC is not seen in birds led Richardson and colleagues to propose that these cells represent an important evolutionary step in mammalian cortical expansion (Richardson et al., 2006; Bergles and Richardson, 2015). Emx2, but not Gsh2 positive cells proliferate in response to demyelinating injury suggesting that despite significant overlap and redundancy these populations are not identical and are likely make distinct contribution for adaptive myelination and behavior later in life (Crawford et al., 2016). Further, differences in OPC composition between Gsh2 ventrally and Gsh2/Emx2 dorsally (Kessaris et al., 2006) might give rise to subtle differences in myelination along the dorsal-ventral axis (Bechler et al., 2018). The dynamic changes in OPC composition during the perinatal period make these processes good candidates for environmental perturbations including EA (**Figure 3B**). However, to the best of our knowledge the effects of EA on OPC composition during the postnatal period has not been investigated yet.

Strict developmental cues instruct OPC to exit the cell cycle and differentiate into short-lived pre-myelinating oligodendrocytes that give rise to mature myelin producing oligodendrocytes (**OL**) or undergo apoptosis (**Figure 4**). Roughly 20–50% of the pre-myelinating oligodendrocytes undergo apoptosis during development, a decision that is tightly linked with their ability to contact and myelinate active axons (Barres et al., 1992; Trapp et al., 1997; Mitew et al., 2018). The rapid cell cycle and high levels of OPC apoptosis are unique features of developmental myelination and are order of magnitude lower in adult rodents (Barres et al., 1992; Mitew et al., 2018) and practically negligible in adult humans (Yeung et al., 2014). The high rate of OPC apoptosis during development appears to be driven by a competition for limited survival cues that ensure selective myelination of active axons (Barres et al., 1992; Mitew et al., 2018).

Signals such as Neuregulin 1 (NRG1), adenosine, glutamate, and BDNF, expressed and secreted from activated axons play a critical role in this differentiation process, **Figure 4** (Stevens et al., 2002; Makinodan et al., 2012; Gibson et al., 2014; Wake et al., 2015; Mitew et al., 2018; Geraghty et al., 2019). The tight link between neuronal activation and OPC differentiation makes this process highly sensitive to perturbations induced by EA. For example, Makinodan et al. (2012) has shown that reduced Neuregulin 1-Erbb3 signaling is responsible for abnormal myelination in the PFC in socially deprived juvenile mice and Teissier et al. (2020) has established a causal link between neuronal activity and OPC maturation in a rodent model of EA (**Table 1**).

During development, the brain wires itself by first casting an exuberant rudimentary network and then selecting functional connections and actively eliminating non-functional redundant connections (De Leon Reyes et al., 2020; Pan and Monje, 2020). This process of elimination takes place during a specific period of development, known as the critical period, and is difficult to reverse later in life (McGee et al., 2005; Kaffman and Meaney, 2007; Pan and Monje, 2020). Although still somewhat speculative, a growing body of work has suggested that myelination plays a critical role in promoting circuit refinement during critical or sensitive periods of development. For example, myelin's ability to increase conduction velocity and enhance metabolic support in activated axons is likely to promote the retention of myelinated circuits during development (Saab et al., 2013; Chorghay et al., 2018). Further, the ability of myelin to restrict

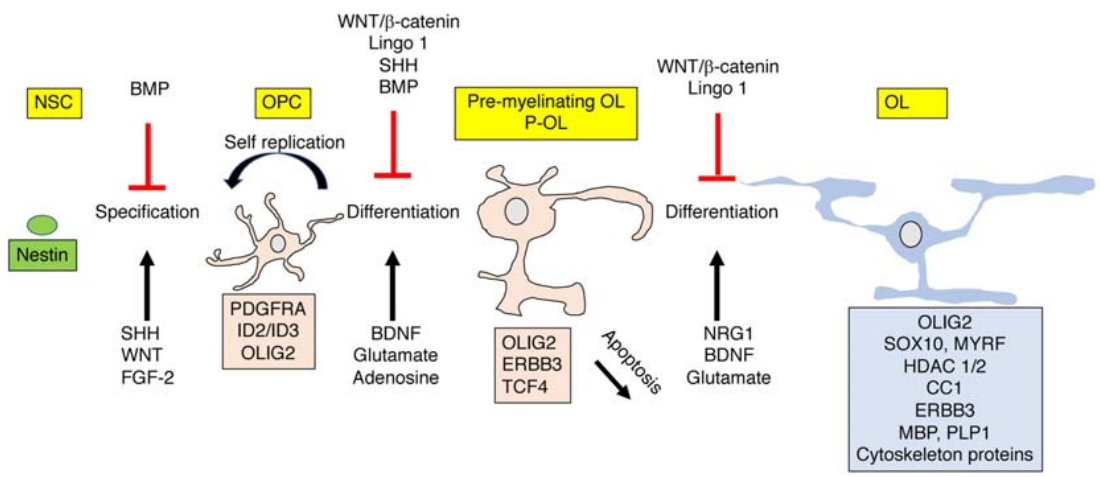

FIGURE 4 | OPC differentiation and apoptosis are highly regulated by external signals. Nestin positive polypotent NSC differentiate into lineage specific OPC that give rise to pre-myelinating (P-OL) or undergo apoptosis. P-OL then differentiate into mature OL. These processes are tightly controlled by diffusible and non-diffusible molecules expressed by active or non-active axons. These instructive molecules can promote (black ↑) or inhibit (red ⊥) these processes by altering gene expression in these cells (shown as a box with matching color). Genes and proteins shown represent only a partial list of key molecules discussed in the text. For more comprehensive reviews see Mitew et al. (2014), Foster et al. (2019). NSC, polypotent neural stem cell; OPC, oligodendrocyte progenitor cells; P-OL, pre-myelinating oligodendrocytes; OL, mature oligodendrocytes.

axonal sprouting may also enhance the selection of myelinated axons by reducing competition for downstream targets (McGee et al., 2005). Ablating myelin during the first 2 weeks of life not only reduces axonal diameter in the corpus callosum, but significantly reduces the heterogeneity in axonal diameter size, consistent with the notion that it promotes the selection of functional circuits (Jalabi et al., 2005). Network properties such as increased global efficiency and a reduction in small-worldness are markers of normal neurodevelopment (Huang et al., 2015) and improved cognition (Bullmore and Sporns, 2009; Huang et al., 2015; Bassett and Bullmore, 2017; Farahani et al., 2019) and are tightly linked with white matter development (Hagmann et al., 2010). Abnormal myelination may therefore account for the reduced global efficiency and increase in small-worldness seen in humans and mice exposed to EA (Ohashi et al., 2019; White et al., 2020). Although appealing and supported by indirect evidence additional work is needed to substantiate and clarify the role that myelination plays in selecting functional circuits during development (**Figure 5**).

One remarkable feature of myelin maturation in adult rodents is the presence of large number of unmyelinated axons accompanied by a sizable pool of OPC in many brain regions (Mitew et al., 2018; Steadman et al., 2020). This unique feature provides an important source of plasticity in adulthood that appears to be impaired by some forms of EA (Yang et al., 2017; Teissier et al., 2020). At the same time, agents that promote *de novo* myelination during development or even later in life may be used to compensate for earlier myelination and behavioral deficits caused by EA. Although significant *de novo* myelination has been seen in adult humans, it seems to be driven mainly by expansion of myelin in mature OL rather than a recruitment of OPC (Yeung et al., 2014). This difference in adaptive myelination may have important implications for challenges in translating successful interventions in adult rodents to humans and will benefit from additional work in non-human primate models of EA (see more on this issue below).

EA CAUSES WHITE-MATTER ABNORMALITIES IN HUMANS, NON-HUMAN PRIMATES AND RODENTS

Human Studies

Reduced corpus callosum size is one of the most consistent findings reported in individuals exposed to EA (Teicher et al., 1997, 2004; De Bellis et al., 1999, 2002; Cohen et al., 2006; Kitayama et al., 2007; Rusch et al., 2007; Andersen et al., 2008). These findings were seen in children and adults indicating that EA impairs normal myelin development in a manner that persists into adulthood. More recent work with diffusion MRI (dMRI) has extended the volumetric assessment to identify reduced fractional anisotropy (**FA**) – a measure of water diffusion and microstructural changes – in the corpus callosum of maltreated individuals (Jackowski et al., 2008; Paul et al., 2008; Teicher et al., 2010; Frodl et al., 2012; Huang et al., 2012; Lu et al., 2013; Bick et al., 2015; Rinne-Albers et al., 2016; McCarthy-Jones et al., 2018). Alterations in FA were also reported in other white matter tracks including the cingulum bundle, corona radiata, superior longitudinal fasciculus, inferior frontal-occipital fasciculus, fornix and the uncinate fasciculus, but these findings have been less reproducible (see **Table 1**). Bick and colleagues found that adopting children that were institutionalized during the first two years of life into supportive families was able to reverse some but not all of the white matter abnormalities at ages 8–11 (Bick et al., 2015). Interestingly, reduced FA in the corpus callosum persisted even after adoption suggesting that these changes might require longer time to correct or may not be fully correctable later in life (**Table 1**).

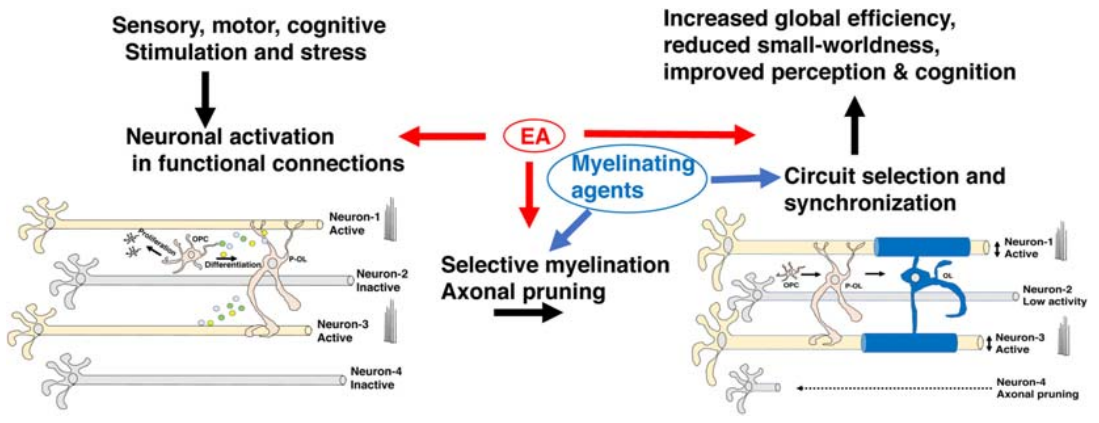

FIGURE 5 | Myelin promotes the selection of functional circuits during development. Environmental stimulation or deprivation early in life leads to changes in neuronal activity in the developing brain. Increased activation in appropriately connected functional units (neurons 1 & 3) but not in neurons 2 & 4 leads to preferential myelination, enhanced metabolic support, and axonal growth in neurons 1 & 3 at the expense of neuron 2. Neuron 4 undergoes axonal pruning commonly seen during a critical period of transcallosal-projection development and is irreversible later in life. Adaptive myelination coupled with axonal pruning promotes synchronization of functional circuits and maturation of network properties (e.g., increased global efficiency and reduced small-worldness) that improve cognition and perceptual accuracy. Exposure to EA interferes with different aspects of this process and can potentially be reversed by myelinating agents. EA, early adversity; OL, mature oligodendrocyte; OPC, oligodendrocyte progenitor cell; P-OL, pre-myelinating oligodendrocytes.

Using postmortem analysis, Tanti et al. reported increased density of mature oligodendrocytes in the ventro-medial prefrontal cortex of individuals with a history of EA and depression who died by suicide (DS-EA) compared to individuals with a history of depression who died by suicide but had no history of EA (DS) or a control group. The functional implications of this increase in the number of mature oligodendrocytes are yet to be clarified given that other markers of myelination (e.g., PLP, CNP, MAG, MOG, MOBP) were unaffected by or even decreased (e.g., MBP) in both DS-EA and DS groups compared to the control groups (Tanti et al., 2018), see also **Figure 4**. Moreover, as reviewed above, there is currently limited evidence for microstructural alterations in white matter prefrontal cortex in individuals with a history of EA.

Clinical Significance

The corpus callosum is the largest white matter fiber track in the brain connecting the left and the right hemispheres and is responsible for the integration of sensory and motor information, executive function, language acquisition, and the management of emotional and social responses (Fryer et al., 2008; De Leon Reyes et al., 2020). The corpus callosum is organized in a homotypic manner in which equivalent areas in both hemispheres are connected. This organization ensures a specific flow of information across the caudal-rostral axis with sensory information located at the most caudal regions and prefrontal cortex executive flow of information across the rostral end (**Figure 3A**). Transcallosal projections undergo significant activity-dependent pruning during postnatal development and are therefore highly sensitive to environmental changes including EA (De Leon Reyes et al., 2020), see neuron 4 in **Figure 5**. Myelination of transcallosal projections starts at the caudal end reflecting early maturation of somatosensory and language capabilities early in childhood and more complex social and executive functions in late adolescence early adulthood (Bechler et al., 2018; De Leon Reyes et al., 2020). Additional work is needed to clarify whether excessive pruning of transcallosal projections is responsible for the corpus callosum abnormalities seen in individuals exposed to EA and whether abnormal myelination is actively contributing to this process. Addressing these questions will have important implications for the feasibility of reversing EA-mediated myelin abnormalities in the corpus callosum.

Alterations in CC and other white matter tracks are commonly seen in a broad range of psychiatric disorders (Fields, 2008; McCarthy-Jones et al., 2018; De Leon Reyes et al., 2020), many of which are likely to be attributed to EA (Teicher and Samson, 2016). The clinical significance of these abnormalities is a critical question that is yet to be clarified, especially given that FA changes are also seen in asymptomatic adult individuals with a history of EA (McCarthy-Jones et al., 2018). Moreover, some individuals with a complete absence of corpus callosum show little clinical deficits while others show very significant motor, social, and cognitive deficits (De Leon Reyes et al., 2020). Addressing the functional significance of these white matter abnormalities in humans is difficult, if not impossible, but important insights can be gained from work in animals.

Non-human Primates

Reduced corpus callosum size and FA have also been reported in non-human primates exposed to EA (Sanchez et al., 1998; Coe et al., 2002; Jackowski et al., 2011; Howell et al., 2013), and **Table 1**. For example, 1 year old rhesus monkeys (*Macaca mulatta*) that were singly caged from 2 to 6 months and then group-housed for 6 months or maintained isolated for the entire 10 months (i.e., 2–12 months) showed reduced corpus callosum volume that was most pronounced in posterior regions. Reduced white matter volumes were also seen in the prefrontal cortex and parietal cortex and were associated with impaired acquisition of the delayed-non-matching to sample task (Sanchez et al., 1998). No differences were seen in corpus callosum size between animals that were group-housed during the last 6 months and those that were isolated for the entire 10 months (Sanchez et al., 1998). These findings are consistent with the adoption findings reported by Bick et al. (2015) and the notion that early deprivation causes long-term changes in corpus callosum size that are difficult to reverse. It will be interesting to test whether myelinating agents such as Clemastine fumarate can reverse some of the myelin and cognitive deficits seen in singly caged monkeys. This work will also clarify whether simply augmenting myelination can correct cognitive and behavioral abnormalities even in the absence of appropriate social stimulation.

Reduced corpus callosum size was also reported in 5 year old Bonnet monkeys (Macaca radiata) exposed to variable foraging demands between the ages of 2–6 months (Jackowski et al., 2011). This paradigm exposes mother-infant dyads to periods of time in which food availability is uncertain leading to increased maternal stress, insecure attachment, and increased emotional liability in the offspring (Andrews and Rosenblum, 1991). Finally, exposure to maternal abuse early in life was associated with reduced FA in the corpus callosum of adolescent rhesus monkeys that was correlated with elevated corticosterone levels measured at the peak of the abusive behavior when the infants were 1 month old (Howell et al., 2013).

In summary, non-human primates exposed to different types of EA show similar reduction in corpus callosum size and FA to those reported in humans (**Table 1**). Additional work is needed to clarify whether changes in corpus callosum size are primarily due to reduced number of callosal projections and whether abnormal myelination is simply a consequence of this reduction or a critical factor in destabilizing and eliminating these projections during a critical period of development. Work with non-human primates can also clarify whether myelinating agents can reverse some of the myelin abnormalities and cognitive deficits associated with EA and determine the age in which the administration of these agents is most efficacious (**Figure 5**). Work with non-human primate models of EA is crucial for addressing questions about timing for interventions because of important differences in the mechanisms responsible for adult *de novo* myelination in rodents and humans (Yeung et al., 2014).

Studies in Rodents

Recent work in rodents provides some important insights into possible mechanisms by which EA alters myelination

and complex behavior (summarized in **Table 1**). For example, using PLP-eGFP mice, Makinodan and colleagues found that isolating juvenile mice after weaning (P21-35) caused abnormal maturation of oligodendrocytes in the prefrontal cortex (**PFC**). Reduced activation of the Erbb3 receptor on oligodendrocytes, most likely due to reduced levels of its axonal ligand NRG1, were responsible for the abnormal myelination in the PFC and the impaired performance in the non-matching to place task. The use of PLP1-EGFP mice is an important technical detail because it revealed reduced ramification of myelin containing processes without affecting the density of these cells (Makinodan et al., 2012). Interestingly, abnormal myelination and behavioral deficits were seen when mice were socially isolated between P21-35, but not after P35. Group housing mice that were singly housed during the juvenile period with other socially isolated mice later in life did not reverse the myelin or behavioral deficits and similar behavioral and myelin deficits were seen in mice in which the Erbb3 receptor was eliminated from oligodendrocytes during the juvenile period (Makinodan et al., 2012). Interestingly, both the myelin and the behavioral deficits were reversed if previously isolated mice were later group-housed with control mice that were never isolated (Makinodan et al., 2017). Together these findings suggest that adequate levels of social contact during the juvenile period is necessary for Erbb3 mediated myelination and normal PFC function and that appropriate social milieu in adulthood can correct earlier deficits. It is currently unclear if the reduction in NRG1 levels seen in socially isolated mice is due to social deprivation and/or stress, why Erbb3 is no longer necessary for myelin formation after the sensitive period ended, and what aspects of social contact later in life are necessary to restore normal myelination and to improve PFC function later in life.

Yang et al. (2017) have shown that 3 h maternal separation (**MS**) during the first 3 weeks of life reduces myelination in the PFC in rats. These deficits were seen after weaning (P21) and in adult animals (P60) and were associated with reduced levels of mature oligodendrocytes and increased levels of OPC suggesting that MS impaired *de novo* myelination in adulthood. The blockade in OPC maturation was associated with reduced levels of the histone deacetylases 1 and 2 (HDAC1/2), and increased expression of WNT-related genes that blocked OPC differentiation (e.g., β-catenin, ID2, ID3, see also **Figure 4**). Administration of the HDAC1/2 inhibitor valproic acid (VPA) from P2-23 caused similar deficits in myelination and behavioral abnormalities seen in rats exposed to MS, whereas injecting the WNT antagonist XAV939 during the first 3 weeks of life reversed the myelination and behavioral abnormalities seen in MS. Together, these findings suggest that MS causes long-term increase in WNT signaling that blocks OPC differentiation and *de novo* myelination in the adult PFC (**Figure 2**), providing one of the most compelling mechanistic studies looking at this question in rodents. Importantly, this work is the first to demonstrate that myelinating agents, such as XAV939, can reverse the myelination and behavioral deficits seen in rat model of EA. An important limitation of this work is that it was done using cell lysates from the PFC and provide limited evidence for changes in gene expression in OPC. Moreover, MS did not impair myelination in the corpus callosum which is one of the most robust findings in humans and non-human primates (**Table 1**).

Teissier et al. (2020) found that mice exposed to 3 h split litter MS (**slMS**) during the first 2 weeks caused precocious maturation of OPC in the PFC at P21 and reduced number of OPC in the adult PFC of mice exposed to slMS. Precocious maturation of OPC early in life was associated with reduced neuronal activation in the developing PFC of slMS pups. Using chemogenetic activating and inhibitory viruses expressed in the PFC of newborn pups, the authors showed that inhibiting neuronal activity during the first two weeks of life led to precocious myelination in P15 pups, reduced OPC cell number in adulthood and caused similar behavioral deficits to those seen in mice exposed to slMS. Further, activating PFC neurons from P2-14 led to reduced myelination at P15 and exuberant myelination in adulthood that was able to reverse the behavioral deficits seen in slMS mice. The authors proposed that MS suppresses neuronal activation in the developing PFC leading to precocious maturation of OPC and depletion of OPC in adulthood (Teissier et al., 2020). Additional work is needed to clarify whether this reduction in OPC levels impairs adaptive myelination and its contribution to cognitive performance in adulthood. Although the work nicely demonstrates that neuronal inactivation causes similar alterations in myelin and behavioral changes to those seen in maternally separated mice, it is unclear whether the changes in myelination or other non-myelin related processes such as synaptic strength/pruning are responsible for the behavioral outcomes. Second, these findings seem inconsistent with a large body of work showing that OPC proliferation and differentiation increase with neuronal activation rather than neuronal inactivity (Stevens et al., 2002; Gibson et al., 2014; Hines et al., 2015; Mitew et al., 2018; Pan et al., 2020), although this might be a unique feature of the developing PFC and reflect underlying heterogeneity and region specific patterns of myelination discussed above (**Figure 3B**).

Work from several groups has shown that corpus callosum size is highly sensitive to early manipulations including early life stress in the mouse. Using handling, as a model of early stimulation, the Denenberg lab showed increased corpus callosum size in P110 adult rats exposed to handling during the first three weeks of life (**Table 1**). Increase in corpus callosum size was seen in males, while female rats showed reduced corpus callosum size and these rearing effects were no longer present in P225 rats (Berrebi et al., 1988). Handling is considered a form of early enrichment that is due to enhanced sensory stimulation induced by touching the pups and exposing them briefly to novel environmental milieu (Reeb-Sutherland and Tang, 2012). In addition, brief removal of the pups causes an increase in maternal care when the pups are reunited with the dam which is known to be a potent stimulator many aspects of neurodevelopment (Kaffman and Meaney, 2007; Couto-Pereira et al., 2016). This work is consistent with the notion that callosal projections and corpus callosum size are highly sensitive to sensory input early in life (**Figure 5**).

Duque et al. (2012) exposed mice pups to prolong daily maternal separation (4–8 h) from P2-P17, followed by early weaning at P17, a procedure they named maternal separation

with early weaning (***MSEW***). Using this approach, they found a significant reduction in both gray and white matter that were more pronounced in the left hemisphere. They further confirmed their stereological and histological findings using dMRI showing reduced FA in the rostral and medial sections of the corpus callosum (Carlyle et al., 2012; Duque et al., 2012). Reduced myelination in the corpus callosum was associated with increased density of Olig2-positive cells (a marker of both OPC and OL, **Figure 4**) but reduced expression of genes expressed in mature OL suggesting that MSEM blocked OPC differentiation in the corpus callosum (Bordner et al., 2011; Duque et al., 2012). These findings are the first example showing reduced CC size in a rodent model of ELS. The translational utility of these changes is yet to be clarified because they exclusively affect the left hemisphere (Duque et al., 2012), a finding that has not been reported in humans, non-human primates or other mouse models. For example, using unbiased whole brain voxel analysis, White et al. (2020) found reduced volume and FA in the rostral regions of the corpus callosum in mice exposed to complex early life stress (White et al., 2020). These changes were seen in both males and females and were present in both the left and the right hemispheres. These findings are the first to use human imaging tools to confirm reduction is corpus callosum size and FA in a mouse model of ELS using unbiased whole-brain voxel analysis with rigorous correction for multiple comparisons (**Table 1**).

In summary, different paradigms of EA models in mice have been associated with abnormal myelination in adulthood. Most of the work in mice has focused on myelination in the PFC, but reduced corpus callosum size and FA have also been reported in some models. The available mechanistic studies suggest activation of WNT/β-catenin signaling impairs OPC differentiation in the adult PFC (Yang et al., 2017) while other forms of EA suppress neuronal activation in the developing PFC leading to precocious OPC maturation and OPC depletion later in life (Teissier et al., 2020). Importantly, emerging evidence suggests that myelinating agents can reverse the white matter and behavioral abnormalities seen in mice exposed EA or prolonged stress in adulthood (Liu et al., 2016; Yang et al., 2017).

CONCLUSION AND FUTURE DIRECTIONS

EA impairs white matter development in a manner that persists later in life in humans, non-human primates and rodents with reduced corpus callosum size and FA as the most consistent findings. Clarifying the mechanisms by which different forms of EA cause long-term changes in corpus callosum development and its impact on brain lateralization and behavior are important questions for translational research. Unfortunately, most of the work in rodents has focused on myelin development in the PFC with relatively few models showing abnormal corpus callosum development. Conversely, the effects of different forms of EA on myelination in the PFC of humans has received little attention and is worth revisiting.

Alterations in neuronal activity in response to deprivation or threat early in life are likely to play an important role in modifying myelin development (**Figure 5**). It is unclear however, whether neuronal activation increases or decreases myelin development. The emerging answer suggests that this might be circuit specific, begging the question of what signals mediate these different responses. Additional work is needed to clarify the role that compacted and uncompacted myelin plays in stabilizing axonal projections and synaptic strengthening during development. The distinction between myelin contribution to conduction velocity and metabolic coupling has not received enough attention with regard to their impact on circuit development and their functional consequences later in life. Differences in the involvement of compacted vs. uncompacted myelin may also explain why seemingly similar alterations in white matter are associated with very different functional consequences (De Leon Reyes et al., 2020).

Additional studies are needed to clarify the mechanisms by which EA reprograms OPC function later in life and how these changes impact adaptive myelination and behavior later in life. Finally, the availability of large number of myelinating agents (Murphy and Franklin, 2017) and genetic tools to block adaptive myelination (Makinodan et al., 2012; Pan et al., 2020) enable the field to test whether agents that increase myelination can reverse the myelination and cognitive deficits seen in animals exposed to EA, which circuits are more responsive, and when is the best time to apply them. Promising findings in rodents and especially in non-human primates will likely pave the way for new highly needed clinical interventions in humans.

AUTHOR CONTRIBUTIONS

RI reviewed the literature, wrote, and edited the manuscript. AK conceptualized the content, reviewed the literature, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Early Rearing Conditions Affect Monoamine Metabolite Levels During Baseline and Periods of Social Separation Stress: A Non-human Primate Model (*Macaca mulatta*)

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Wood EK, Gabrielle N, Hunter J, Skowbo AN, Schwandt ML, Lindell SG, Barr CS, Suomi SJ and Higley JD (2021) Early Rearing Conditions Affect Monoamine Metabolite Levels During Baseline and Periods of Social Separation Stress: A Non-human Primate Model (Macaca mulatta). Front. Hum. Neurosci. 15:624676. doi: 10.3389/fnhum.2021.624676

A variety of studies show that parental absence early in life leads to deleterious effects on the developing CNS. This is thought to be largely because evolutionary-dependent stimuli are necessary for the appropriate postnatal development of the young brain, an effect sometimes termed the "experience-expectant brain," with parents providing the necessary input for normative synaptic connections to develop and appropriate neuronal survival to occur. Principal among CNS systems affected by parental input are the monoamine systems. In the present study, N = 434 rhesus monkeys (233 males, 201 females) were reared in one of two conditions: as mother-reared controls (MR; n = 269) or without adults with 24-h access to same-aged peers (PR; n = 165). When subjects were six-months-old, they underwent a separation paradigm involving 4, sequential, four-day social separations from their mothers or peers, with each separation followed by three-day reunions with their mothers or their peers. Prior to the separation paradigm, baseline cisternal CSF samples were obtained, as well as at the end of each the four social separations, and after final separation, during a recovery period. CSF was assayed for concentrations of monoamine metabolites and a blood sample was genotyped for the serotonin transporter (5-HTT) genotype. Replicating earlier landmark findings, PR subjects with the *s* allele exhibited lower baseline concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), when compared to PR subjects homozygous for the *L* allele. MR subjects were undifferentiated by genotype. PR subjects exhibited lower CSF 5-HIAA concentrations during baseline, but higher CSF 5-HIAA during social separations, when compared to MR subjects. There were rearing effects for the dopamine metabolite homovanillic acid (HVA) and for the norepinephrine

metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), with PR subjects showing higher HVA and lower MHPG when compared to MR subjects. These findings indicate that there are long-term deficits in the response of monoamines following early maternal absence. The results of this study confirm and extend earlier findings that early parental absence has deleterious consequences for the development of the monoamine systems, and that these consequences are modulated by the 5-HTT genotype.

Keywords: dopamine, early rearing, monoamine metabolites, norepinephrine, rhesus macaque, serotonin, serotonin transporter genotype, social separation

INTRODUCTION

In primates, most cortical brain development occurs postnatally (Bakken et al., 2016). Shortly before and immediately following birth, infants experience an explosive process of synapse formation, known as exuberant synaptogenesis (Eayrs and Goodhead, 1959; Altman, 1972; Wang et al., 2009; Fox et al., 2010; Naskar et al., 2019). This period is followed by systematic pruning of the overabundant connections, along with synaptic strengthening in functional neuronal pathways, with both potentiated by experience (Huttenlocher and Dabholkar, 1997; Hashimoto and Kano, 2003; Fox et al., 2010). Studies show that mothers play a critical role in this process of synaptic maintenance and pruning, a process necessary for normative brain development (Sánchez et al., 1998; Scher et al., 2009; Fox et al., 2010; Pollak et al., 2010; Nelson et al., 2011; Schneider et al., 2012). Developmental studies suggest that parental sensitivity is necessary for normative neurobiological development to occur, providing the right input at the right time, something Greenough and colleagues referred to as the "experience-expectant brain" (1987).

Without the appropriate social and physical stimuli that are typically provided by a sensitive caregiver, the "experience-expectant brain" shows marked deficits. For example, studies of humans raised in institutions exhibit fewer synaptic connections relative to their typically-reared peers, resulting in smaller brains (Dobrova-Krol et al., 2008), reduced white matter (Eluvathingal et al., 2006; Mehta et al., 2009), reduced functional connections between structures in the limbic system (Chugani et al., 2001; Daniels et al., 2013; Kumar et al., 2014), increased amygdalae volume (Rutter et al., 2007; Mehta et al., 2009; Tottenham et al., 2010), and decreased corpus callosum volume (Mehta et al., 2009). They also tend to show a dysregulated stress response (Gunnar et al., 2001; Tarullo and Gunnar, 2006). While Nelson et al. (2011) makes the case that it is difficult to tease out the deficits resulting from impoverished early environments and the deficits resulting from poor social experiences, Studies of children with less severe early experiences, who develop in homes with more stimulating surroundings but whose mothers are less responsive to their infants' needs, for instance, mothers who suffer from depression, also show deficits in CNS development and functioning (Ashman and Dawson, 2002; Pruessner et al., 2004; Qiu et al., 2015); albeit, not as extreme as those seen in institutionally-reared children.

While it is difficult to measure neurotransmission and monoamine functioning in children, studies suggest that individuals who were abused in childhood demonstrate higher concentrations of dopamine than controls (de Bellis et al., 1994, 1999; Egerton et al., 2016), as do individuals reporting low maternal care (Pruessner et al., 2004) or neglect (Roy, 2002). Childhood abuse is also associated with low serotonin transporter binding (Miller et al., 2009) and studies suggest that early life neglect and abuse are linked with impaired serotonin functioning (Roy, 2002; Miller et al., 2009). Maltreatment is also associated with elevated norepinephrine concentrations (de Bellis et al., 1999; Otte et al., 2005). Some studies have assessed the role of the early environment on neurotransmission in the context of the serotonin transporter gene (5-HTT), which has two major variants: a more frequently occurring long allele (*L*), and a more recent, less frequently occurring short allele (*s*). The *s* allele of 5-HTT genotype is associated with low transcriptional efficiency, resulting in reduced serotonin expression (Lesch et al., 1996; Little et al., 1998). In humans, studies show that the *L* allele of the 5-HTT genotype confers resilience to maltreatment (Caspi et al., 2010) and that it has a protective effect against the development of insecure attachment in children who were institutionalized as infants (Bakermans-Kranenburg et al., 2012; Humphreys et al., 2015). However, such early rearing aberrations lack experimental control and are often intertwined with parental-child genetic risk.

Due to the difficulties experimentally studying the role of early experience on the brain in human subjects, translational animal models are frequently used to study the impact of parents on infant development. In these models, early rearing conditions can be experimentally-randomized and the environment can be controlled. There is an extensive history of using rhesus monkeys (*Macaca mulatta*) to model the importance of early maternal care on normative development (Harlow, 1958; Suomi, 1995; Kuhn and Schanberg, 1998). Rhesus monkeys are ideally-suited for these types of experiments as they show a high degree of genetic similarity to humans (Gibbs et al., 2007), which is particularly important because, like humans, rhesus monkeys possess an analogous biallelic 5-HTT genotype (Bennett et al., 2002). As in humans, the mother-infant attachment relationship is critical for normative development to occur (Harlow, 1958, 1969); indeed, attachment theory was based, at least in part, on Harlow's studies of rhesus monkeys (van der Horst et al., 2008; van Rosmalen et al., 2020). Rhesus monkeys also show parallel developmental periods (Machado and Bachevalier, 2003; Suomi, 2005), although at an

accelerated rate (Roth et al., 2004), allowing researchers to assess developmental outcomes in a relatively rapid time-frame.

To study the effects of caregivers on infant outcomes, the rearing paradigm most commonly used in rhesus monkeys involves randomly assigning infants to one of two conditions during the first 6 months of life. The experimental group includes peer-reared subjects (PR), a condition where infants are removed from their mothers at birth, hand-reared in a neonatal nursery for 30 days, and then housed together in groups of 2–4 without adults. The controls are reared by their mothers [mother-reared (MR)] in social groups comprising of same-aged infant peers, their mothers, and two adult males, a social condition that approximates the natural rhesus social setting. While PR monkeys are provided ample opportunity for social interactions and live in physical environments that parallel MR subjects, research shows that PR monkeys exhibit altered neurochemical measures (Higley et al., 1991b, 1996, 1992; Clarke et al., 1996; Shannon et al., 2005) and neuroanatomical outcomes (Sánchez et al., 1998; Spinelli et al., 2009, 2010), although the sample sizes for these studies are relatively small, and the majority of these studies have generally not assessed the impact of early maternal absence on neurotransmission as it relates to chronic stress and even less often during a recovery period following the stressors.

The central monoamine system of the rhesus monkey parallels humans in many ways, showing similar resting activity and increased turnover in response to acute and chronic stress (Higley et al., 1991b, 1992, 1996; Clarke et al., 1996; Higley and Bennett, 1999; Shannon et al., 2005). Studies show that serotonin is lower in PR subjects both at baseline (Clarke et al., 1996; Shannon et al., 2005; Spinelli et al., 2010) and during acute stress (Clarke et al., 1996; Bennett et al., 2002), when compared to MR subjects. However, studies have not assessed differences between MR and PR monkeys during conditions of chronic stress nor during post-separation recovery.

Some individuals tend to be relatively resilient to the effects of such early rearing deficits. Caspi and Moffit's landmark study in humans (2002), as well as work published at the same time in rhesus monkeys (Bennett et al., 2002), explain such resilience as resulting from gene X environment interactions. To illustrate, Bennett et al. (2002) showed that PR subjects exhibited reduced CNS serotonin activity, as measured by low cisternal CSF concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). However, when the PR subjects were categorized by serotonin transporter genotype, PR subjects homozygous for *L* allele were relatively unaffected by maternal absence, showing CSF 5-HIAA concentrations that were similar to MR subjects. On the other hand, PR subjects with the *s* allele showed low CSF 5-HIAA concentrations, while MR subjects' CSF 5-HIAA concentrations were undifferentiated by genotype. Such studies suggest that genetic variation may convey resilience to individuals with deleterious early rearing experiences. The present study seeks to replicate this prior finding in an independent sample.

In the present study, the effect of rearing on monoamine metabolite functioning is assessed during baseline, following a repeated social separation stressor, and during post-separation recovery in a large sample of infant rhesus monkeys. Then in an effort to replicate earlier findings (Bennett et al., 2002), subjects are stratified by 5-HTT genotype and their CSF 5-HIAA concentrations are assessed. It was hypothesized that early maternal absence would be associated with altered monoamine measures prior to, during, and following a well-characterized social separation paradigm, and that the 5-HTT genotype would influence stress-induced changes in CSF 5-HIAA.

MATERIALS AND METHODS

Subjects were $N = 434$ infant rhesus monkeys (233 males, 201 females), housed at the National Institutes of Health Animal Center in Poolesville, MD, United States. All subjects were randomly assigned to one of two rearing conditions at birth as part of a larger research program: mother-reared controls (mothers; $n = 269$) or reared with same-aged peers in the absence of adults ($n = 165$). See Shannon et al. (1998) for a detailed description of rearing conditions. Briefly, MR subjects were in born into and reared in social groups comprised of eight-to-twelve adult females, two adult males, and one-to-two other same-aged infants for the first 6 months of life. This condition approximates the social composition of the wild rhesus monkey, albeit smaller in size, than a typical rhesus monkey troop (Lindburg, 1971). PR subjects were separated from their mothers immediately following birth and hand-reared in a neonatal nursery for the first 30 days of life. Following the first 30 days, PR subjects were housed with constant access to three other similarly-reared age-mates. The physical housing for both MR and PR conditions consisted of indoor-outdoor enclosures (indoor: 2.44 × 3.05 × 2.21 m; outdoor: 2.44 × 3.0 × 2.44 m), with a 12-h (0700–1900) light-dark cycle for the indoor enclosure and natural seasonal light cycles for the outdoor enclosure.

To assure that any rearing effects were not the result of dietary differences, based on an earlier study from our nursery showing the importance of essential amino acids on CNS development and behavior (Champoux et al., 2002), the PR infants were hand-fed a 50:50 mixture of Similac (Ross Laboratories, Columbus, OH, United States) and Primalac (Bio-Serv, Frenchtown, NJ, United States) formula supplemented with physiologically relevant concentrations of DHA (1.0%) and arachidonic acid (1.0%) every 2 h until they reached 30 days of age. From day 30 until 4 months of age, formula was administered at 400 mL/day, and standard high-protein monkey chow was gradually introduced to the diet, and the infants were provided *ad libitum* access to water. At 4 months, the amount of formula provided was reduced to 300 mL/day, and, at 5 months, it was reduced to 200 mL/day. Following the nursery experience, both groups were fed commercial monkey biscuits and fruit once daily, and water was available *ad libitum*.

All protocols and procedures employed in this study were ethically reviewed and approved by the NIH Animal Care and Use Committee before beginning this study. This study was conducted in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals, the United States Public Health Service's Policy on Humane Care and

Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals.

Separation Paradigm

When subjects reached 6 months of age, they were captured and separated from their mothers or their same-aged peers for four sequential, 4-day-long social separations, each followed by 3 days of reunion with their mothers or with their peers. Separation procedures are described in detail elsewhere [see Spinelli et al. (2007)]. Briefly, the social separation paradigm began on Monday afternoon at 1,300 h, with the mothers or the same-aged peers' removal from their respective social group. The subject remained in their homecage with the rest of the social group. On day 5 (Friday), the mother or peers were reunited with the infant in the homecage and a three-day reunion period ensued. This procedure was repeated weekly for a period of 4 weeks.

CSF Sampling

Baseline, separation, and recovery concentrations of CSF were sampled from subjects on eight occasions: *Baseline*—one and 2 weeks prior to the separation paradigm; *Separation Stressor*—on the last day of each of the four social separations (Thursdays, prior to reunion); *Recovery*, one and 2 weeks following the final social separation. See **Figure 1** for a summary of the study timeline. On each occasion, CSF samples (1 mL) were collected from the cisterna magna by needle (22-gauge) and syringe (5-cc) *via* spinal puncture, under ketamine hydrochloride anesthesia (15 mg/kg, IM). To avoid the potential for a movement injury, the unconscious subjects were restrained in a lateral recumbent position, holding the head and hips to assure no movement during CSF removal. Ketamine was administered within 10 min and CSF was removed within 30 min of initial disturbance. Studies indicate that when samples are obtained within 30 min of entering the subjects living quarters, acute ketamine administration has no measurable effect on cisternal CSF monoamine metabolite concentrations in rhesus monkeys (Brammer et al., 1987). Samples were flash frozen in dry ice and stored at −70°C until quantified, as previously described [See Agartz et al. (2003) for additional details].

In brief, samples were thawed and 200-μl aliquots and 20-μl of the internal standard, F-HVA (650 pmol), were placed in 10,000 mW cut-off filters (Amicon, Beverly, MA, United States), which were pre-rinsed with water to remove the glycerol, and centrifuged at 10,600 × *g* for 20 min at 4°C. The column used was a Microsorb Short-One, C-18 (Rainin, Woburn, MA, United States) with a Guard-Pak precolumn containing Nova-Pak, C-18 (Waters, Milford, MA, United States). The buffer was 0.05-M sodium acetate, 0.021-M citrate, and 0.25-mM EDTA, pH 4.4. The organic was 5.5% (v/v) acetonitrile and 2% (v/v) methanol. The flow rate was 0.6 ml/min. Two liters of mobile phase was recirculated. Column temperature was 30°C. Standards of MHPG-hemipiperazinium salt, HVA and 5-HIAA were purchased from Sigma (St. Louis, MO, United States). F-HVA was a gift from Kenneth Kirk (NIDDK, Bethesda, MA, United States). Samples with gross blood contaminations (>2%, as indicated by pink coloration) were excluded. Inter- and intra-assay coefficients of variation were less than 10%.

Blood Sampling and Genotyping

Blood (2 mL) was sampled from subjects using a vacutainer (EDTA) and needle (22-gauge) *via* femoral venipuncture under ketamine hydrochloride anesthesia (15 mg/kg, IM), within 10 min of initial disturbance. Blood samples were genotyped for the 5-HTT genotype using procedures described elsewhere (Barr et al., 2004). The serotonin transporter gene promoter region (rh-5HTTLPR) was amplified from 25 ng of genomic DNA with primers (stpr5, 5′-GGCGTTGCCGCTCTGAATGC; intl, 5′-CAGGGGAGATCCTGGGAGGG) in 15-μL reactions with Platinum™ Taq and the PCR$_X$ Enhancer System kit, according to the manufacturer's protocol (Invitrogen™, Carlsbad, CA, United States). Amplifications were performed on a Perkin Elmer® (Wellesley, MA, United States) thermocycler (9700) with one cycle at 96°C for 5 min, followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 30 s, and a final 3-min extension at 72°C. Amplicons were separated by electrophoresis on a 10% polyacrylamide gel, and the short (*s*, 388 bp) and long (*L*, 419 bp) alleles of the rh5-HTTLPR were identified by direct visualization after ethidium bromide staining. The accuracy of the PCR was further confirmed by cutting the bands, eluting and performing Sanger sequencing. See **Supplementary Figure 1** for a representative gel and **Table 1** for sample size distributions.

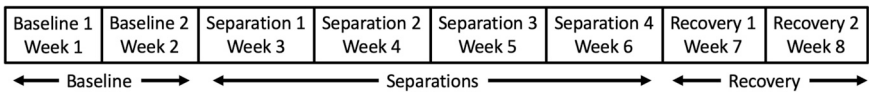

FIGURE 1 | Study timeline. Depicts the timeline for the study, including when all samples were obtained. Baseline 1 included 312 subjects sampled for CSF 5-HIAA concentrations, 258 subjects sampled for CSF MHPG concentrations, and 298 subjects sampled for CSF HVA concentrations. Baseline 2 included 260 subjects sampled for CSF 5-HIAA concentrations, 198 subjects sampled for CSF MHPG concentrations, and 232 subjects sampled for CSF HVA concentrations. Separation 1 included 293 subjects sampled for CSF 5-HIAA concentrations, 260 subjects sampled for CSF MHPG concentrations, and 302 subjects sampled for CSF HVA concentrations. Separation 2 included 239 subjects sampled for CSF 5-HIAA concentrations, 195 subjects sampled for CSF MHPG concentrations, and 236 subjects sampled for CSF HVA concentrations. Separation 3 included 220 subjects sampled for CSF 5-HIAA concentrations, 176 subjects sampled for CSF MHPG concentrations, and 222 subjects sampled for CSF HVA concentrations. Separation 4 included 289 subjects sampled for CSF 5-HIAA concentrations, 244 subjects sampled for CSF MHPG concentrations, and 286 subjects sampled for CSF HVA concentrations. Recovery 1 included 258 subjects sampled for CSF 5-HIAA concentrations, 222 subjects sampled for CSF MHPG concentrations, and 244 subjects sampled for CSF HVA concentrations. Recovery 2 included 213 subjects sampled for CSF 5-HIAA concentrations, 182 subjects sampled for CSF MHPG concentrations, and 215 subjects sampled for CSF HVA concentrations. All samples were obtained on Thursdays at 1300 hours.

Data Analysis

Mixed design, repeated measures analyses of variance (ANOVAs) were utilized to assess the relationship between rearing condition and social separation on CSF monoamine metabolite concentrations.

As they were significantly and positively correlated, the mean of the two *Baseline* samples and the mean of the two *Recovery* samples for the CSF monoamine metabolite concentrations were calculated and used in all analyses. The repeated measure independent variable was *Baseline*, *Separations 1, 2, 3*, and *4*, and *Recovery*. Rearing (MR or PR) was the between-groups independent variable. Unlike an earlier study on a small number of subjects (Higley et al., 1992), there were no sex differences for any of the monoamine metabolite concentrations. A two-way ANOVA was utilized to assess the relationship between 5-HTT genotype and rearing condition on the CSF 5-HIAA concentrations, with rearing condition and 5-HTT genotype as independent variables and CSF 5-HIAA concentrations as the dependent variable. CSF 5-HIAA concentrations were standardized within cohort year, as was done previously [see Bennett et al. (2002)]. All data were analyzed in SPSS, version 26.

RESULTS

Serotonin Metabolite (5-HIAA)

There was a significant main effect of time on cisternal CSF 5-HIAA concentrations [$F(1,173) = 111.43$, $p < 0.0001$]. Further analyses showed that CSF 5-HIAA concentrations obtained during *Baseline* and *Separation 1* were significantly higher than *Separations 2–4* or *Recovery* ($p < 0.0001$). There was also a significant two-way separation-by-rearing condition interaction on CSF 5-HIAA concentrations [$F(1,173) = 5.97$, $p = 0.02$], with MR subjects exhibiting higher CSF 5-HIAA concentrations during *Baseline*, when compared to PR subjects ($p = 0.007$). This effect reversed during the stress of the social separations, with MR subjects showing lower CSF 5-HIAA concentrations during *Separation 1* ($p = 0.04$), *Separation 2* ($p = 0.003$), *Separation 3* ($p = 0.04$), and *Separation 4* ($p = 0.007$), when compared to the PR subjects. PR subjects showed an increase in CSF 5-HIAA concentrations, but PR and MR subjects showed no difference from *Separation 4* to *Recovery* ($p = 0.60$), and neither group returned to *Baseline* levels during *Recovery*. See **Figure 2**.

Norepinephrine Metabolite (MHPG)

There was a significant main effect of time on cisternal CSF MHPG concentrations [$F(1,141) = 10.21$, $p = 0.002$]. Further analyses showed that CSF MHPG concentrations obtained during *Baseline* and *Separations 1 and 2* were significantly higher than during the subsequent separations or the *Recovery* period ($p < 0.05$). There was also a significant main effect of rearing [$F(1,141) = 14.15$, $p < 0.0001$], with MR subjects exhibiting higher overall cisternal CSF MHPG concentrations ($M = 146.74 \pm 2.90$), when compared to the PR subjects ($M = 127.72 \pm 4.14$). MR subjects exhibited higher CSF MHPG concentrations at *Baseline* ($p = 0.02$), *Separation 1* ($p = 0.003$), *Separation 2* ($p = 0.005$), *Separation 3* ($p = 0.003$), *Separation 4* ($p < 0.0001$), and *Recovery* ($p = 0.008$), when compared to PR subjects. See **Figure 3**.

TABLE 1 | Distribution of 5-HTT genotypes across rearing groups.

	LL	*Ls/ss*
MR	133	93
PR	67	27

Includes the sample size and 5-HTT genotype distribution for each of the rearing groups included in the study.

Dopamine Metabolite (HVA)

There was a significant main effect of time on cisternal CSF HVA concentrations [$F(1,175) = 5.85$, $p = 0.02$)], with *Baseline* CSF HVA concentrations significantly higher than concentrations obtained during *Separations 1–4* or the *Recovery* period ($p < 0.0001$). There was also a significant main effect of rearing [$F(1,175) = 8.36$, $p = 0.004$], with MR subjects exhibiting lower overall cisternal CSF HVA concentrations ($M = 1808.74 \pm 27.92$), when compared to PR subjects ($M = 1933.47 \pm 34.84$). MR subjects exhibited lower CSF HVA concentrations at *Separation 1* ($p = 0.005$), *Separation 2* ($p = 0.03$), *Separation 3* ($p = 0.003$), *Separation 4* ($p = 0.04$), and *Recovery* ($p = 0.01$), when compared to PR subjects. MR and PR subjects' CSF HVA concentrations did not differ at *Baseline* ($p = 28$). See **Figure 4**.

5-HTT Genotype and Serotonin Metabolite (5-HIAA)

There was a significant effect of 5-HTT genotype on CSF 5-HIAA concentrations [$F(1,134) = 4.43$, $p = 0.04$], with homozygous subjects exhibiting higher CSF 5-HIAA concentrations on average ($M = 427.10 \pm 5.91$), when compared to subjects with an *s* allele ($M = 396.296 \pm 9.682$). Replicating an earlier study (Bennett et al., 2002), there was also a significant two-way rearing-by-5-HTT-genotype interaction on CSF 5-HIAA concentrations [$F(1,134) = 4.29$, $p = 0.04$], with PR subjects with an *s* allele exhibiting lower CSF 5-HIAA concentrations on average ($M = 362.12 \pm 16.43$), when compared to MR subjects with an *s* allele ($M = 430.47 \pm 10.25$), or to homozygous MR ($M = 430.66 \pm 7.73$) or PR subjects ($M = 423.54 \pm 8.94$). See **Figure 5**.

DISCUSSION

In line with the hypotheses, the results showed that early rearing experiences play a role in baseline monoamine metabolite concentrations and that monoamine metabolite concentrations are modulated by early rearing experiences during stress-inducing social separations and during a recovery period. This paper also replicates, for the first time the seminal findings of a gene-by-environment interaction effect on central

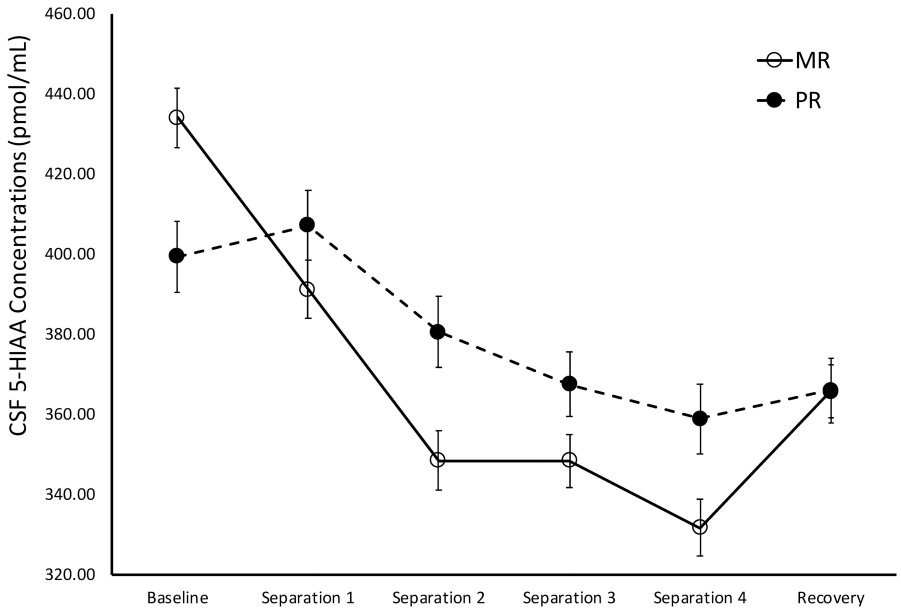

FIGURE 2 | Rearing effects on baseline and stress-induced CSF 5-HIAA concentrations. Depicts the relationship between rearing condition and CSF 5-HIAA concentrations during *Baseline, Separations 1–4*, and *Recovery*. There was a significant main effect of time on CSF 5-HIAA concentrations [$F(1,173) = 111.43$, $p < 0.0001$], with CSF 5-HIAA concentrations higher during *Baseline* and *Separation 1*, when compared to *Separations 2–4* or *Recovery*. There was also a significant two-way separation-by-rearing condition interaction on CSF 5-HIAA concentrations [$F(1,176) = 6.05$, $p = 0.02$], with MR subjects exhibiting higher CSF 5-HIAA concentrations during *Baseline*, but lower CSF 5-HIAA concentrations during *Separations 1–4*, when compared to PR subjects. MR subjects are represented by white circles and solid lines, PR subjects are represented by black circles and dashed lines.

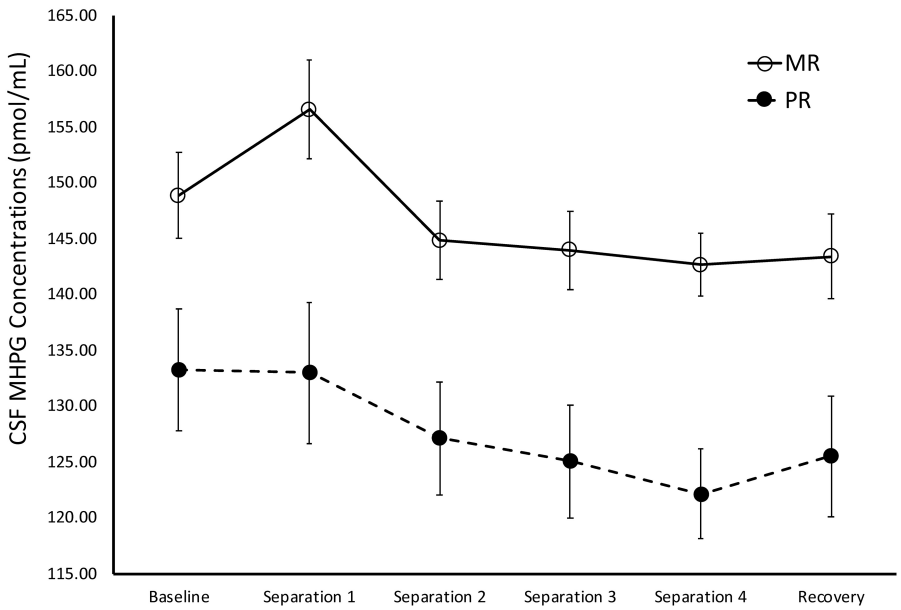

FIGURE 3 | Rearing effects on baseline and stress-induced CSF MHPG concentrations. Depicts the relationship between rearing condition and CSF MHPG concentrations during *Baseline*, *Separations 1–4*, and *Recovery*. There was a significant main effect of time on CSF MHPG concentrations [$F(1,141) = 10.21$, $p = 0.002$], with CSF MHPG concentrations higher during *Baseline* and *Separations 1* and *2*, when compared to *Separations 3* and *4* or *Recovery*. There was also a significant main effect of rearing [$F(1,141) = 14.15$, $p < 0.0001$], with MR subjects exhibiting higher CSF MHPG concentrations at *Baseline*, *Separations 1–4*, and Recovery, when compared to PR subjects. MR subjects are represented by white circles and solid lines, PR subjects are represented by black circles and dashed lines.

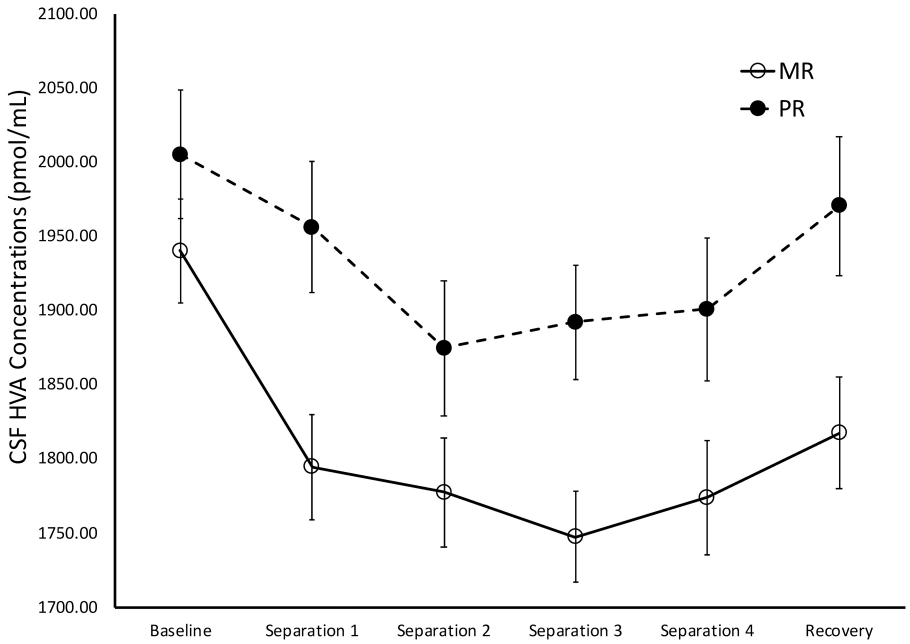

FIGURE 4 | Rearing effects on baseline and stress-induced CSF HVA concentrations. Depicts the relationship between rearing condition and CSF HVA concentrations during *Baseline*, *Separations 1–4*, and *Recovery*. There was a significant main effect of time on CSF HVA concentrations [$F(1,175) = 5.85$, $p = 0.02$], with CSF HVA concentrations higher at *Baseline*, when compared to *Separations 1–4* or *Recovery*. There was also a significant main effect of rearing [$F(1,175) = 8.36$, $p = 0.004$], with MR subjects exhibiting lower CSF HVA concentrations at *Separations 1–4* and Recovery, when compared to PR subjects. MR subjects are represented by white circles and solid lines, PR subjects are represented by black circles and dashed lines.

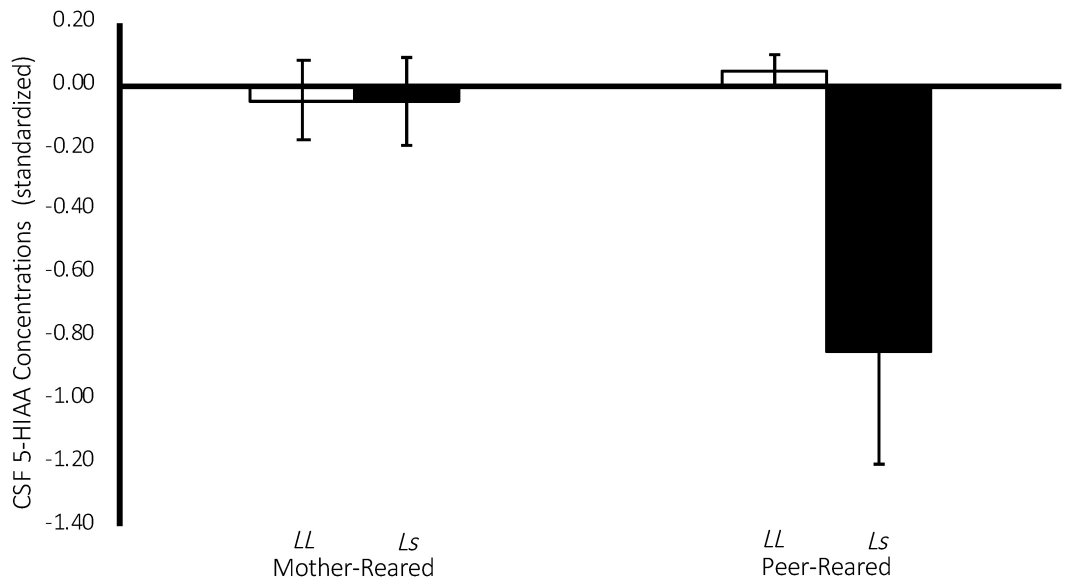

FIGURE 5 | Interaction between 5-HTT genotype and rearing condition on CSF 5-HIAA Concentrations. There was a significant effect of 5-HTT genotype on CSF 5-HIAA concentrations [$F(1,134) = 4.43$, $p = 0.04$], with homozygous subjects exhibiting higher CSF 5-HIAA concentrations on average, when compared to subjects with an *s* allele. There was also a significant two-way rearing-by-5-HTT-genotype interaction on CSF 5-HIAA concentrations [$F(1,134) = 4.29$, $p = 0.04$], with PR subjects with an *s* allele exhibiting lower CSF 5-HIAA concentrations on average, when compared to MR subjects with an *s* allele, or to homozygous MR or PR subjects. MR subjects are represented in white, PR subjects are represented in black.

serotonin metabolite levels, with PR subjects with the *s* allele exhibiting lower CSF 5-HIAA concentrations, when compared to homozygous PR subjects or to MR subjects with either genotype (Bennett et al., 2002).

MR subjects exhibited significantly higher CSF 5-HIAA concentrations before the stress-inducing separations, when compared to the PR subjects, consistent with a large number of earlier studies showing that baseline CSF 5-HIAA concentrations are modulated by early rearing experiences (Higley et al., 1992, 1996; Clarke et al., 1996; Shannon et al., 2005; Spinelli et al., 2010). However, during the stress-inducing social separations, the rearing effect on CSF 5-HIAA reversed, with the CSF 5-HIAA concentrations of MR subjects falling below that of the PR subjects, suggesting that the effects of early rearing experiences on the serotonin system are affected differentially by stress (see **Figure 2**). One potential interpretation of this finding is that, by nature of their early experiences, PR subjects develop a serotonin system that is less sensitive to changing situations, while MR subjects' serotonin systems are more sensitive to stress-inducing conditions. As a percentage of baseline, the MR controls showed a much greater decline in cisternal CSF 5-HIAA concentrations across repeated separations than did the PR subjects (MR: 16% decline in cisternal CSF 5-HIAA levels; PR: 7% decline in cisternal CSF 5-HIAA levels). Unlike the PR subjects, the MR subjects showed a significant increase in CSF 5-HIAA concentrations during *Recovery*, although they did not completely return to *Baseline* concentrations. Future studies should extend the cisternal CSF sampling to investigate whether the MR subjects returned to their *Baseline* levels if given enough time. Other work from investigating the relationship between early rearing conditions and later life behavior follows a similar pattern: PR subjects show significantly greater alcohol intake, when compared to MR subjects during baseline, but the MR subjects increase their consumption to that of the PR subjects during a social separation stressor, nearly returning to baseline after being reunited with their the cage-mates (Higley et al., 1991a), a compelling similarity, given the relationship between low central serotonin and alcohol consumption in human and non-human primates (Higley et al., 1991a, 1996; LeMarquand et al., 1994).

These results suggest that a mother's influence on her infant's serotonin system not only assures normative levels of serotonin activity, but that, developmentally, mother's maintain an infant's responsiveness and sensitivity to environmental stimuli, particularly to stress. Absent the appropriate maternal input during this sensitive period, PR subjects exhibit lower serotonin activity at *Baseline* and less sensitivity and responsiveness during to stress, as evidenced by a reduced percent change in serotonin metabolite concentrations from *Baseline to Separations 1–4*. While speculative, it is possible that this may be related to the chronic stress that the PR subjects experience. Perhaps as a result of this, PR subjects showed relatively little change in CSF 5-HIAA concentrations when undergoing social separation procedures, while MR subjects, which, with exception to the social separation, live in the constant presence of their mother, exhibit drastic changes in CSF 5-HIAA concentrations. Given that mothers may help maintain their infant's arousal during times of stress, it may be more stressful for MR subjects to undergo separation from their mothers, when compared to PR subjects' separation from their peer groups. While it is beyond the scope of this study to mechanistically explain the relatively greater stress-induced change in CSF 5-HIAA concentrations in the MR subjects when compared to the PR subjects, Clarke et al. (1996) showed that, while MR subjects tend to exhibit stable, trait-like positive correlations in monoamine metabolite concentrations across development, PR subjects exhibit no such correlations. In another study, Kraemer et al. (1989) also showed that the three monoamine metabolites are intercorrelated across development in the MR subjects, but not in subjects reared without their mothers, arguing that the lack of a sensitive mother that reliably provides a secure base to reduce arousal, the hallmark of the attachment figures o individuals with a secure mother-infant attachment, is likely the source of this discrepancy.

During *Baseline*, stress-inducing *Separations*, and *Recovery* conditions, the maternally-deprived PR subjects exhibited significantly lower CSF MHPG concentrations, when compared to MR subjects (see **Figure 3**). These findings are in contrast to earlier work showing that early childhood maltreatment and trauma are associated with elevated norepinephrine concentrations (de Bellis et al., 1999; Otte et al., 2005), and illustrate the point discussed earlier that not all stressors are the same in their effect. Similarly, the PR subjects exhibited significantly higher CSF HVA concentrations during the stress-inducing *Separations* and *Recovery* conditions, when compared to MR subjects (see **Figure 4**). These findings are corroborated by earlier work showing that, when compared to children raised in homes where parents are sensitive to their infants' emotional needs, abused and neglected children exhibit higher urinary dopamine concentrations (de Bellis et al., 1994, 1999; Roy, 2002; Egerton et al., 2016), as do children who experienced poor early maternal care, as measured by dopamine binding in PET assessment (Pruessner et al., 2004). These findings suggest that the effect of maternal absence leads to an atypical stress-response and potentially, an attenuated reward system. Using independent evolutionary theoretical models, Belsky (2002) and Ein-Dor et al. (2010) suggest that aberrations from typical development are not necessarily abnormal, but instead may be adaptations to the demands of the environment. In the case of PR subjects, one interpretation of their deviations from MR subjects in response to the stress of changing situations, is that the maternal absence they experienced early in life may have promoted adaptations to stressful situations, potentially leading to increased vigilance. These findings may also explain why PR subjects show reduced motivation and responses to rewarding stimuli (Paul et al., 2000; Nelson et al., 2009).

These findings suggest that a mother's influence is instrumental in maintaining the interplay and dynamics between and within the monoamine systems. Regardless of the mechanism, overall, it is clear that maternal absence during this formative time has profound effects on the

development of the monoamine systems, consistent with the theory that normative developmental processes are critically dependent on the right input at the right time, as proposed by Greenough et al. (1987). In this case, the right input is from mother, and her importance is punctuated by the differences between the MR and PR subjects in the response of the monoamine systems to stress. To paraphrase the now-classic words of Hebb (2005): in the absence of the right input (sensitive maternal response to arousal), at the right time, the systems failed to fire together, and, thus, failed to wire together.

Replicating earlier work (Bennett et al., 2002), there was a significant 5-HTT-genotype-by-rearing-condition interaction on CSF 5-HIAA concentrations, such that PR subjects with an *s* allele exhibited lower CSF 5-HIAA concentrations on average, when compared to MR subjects with an *s* allele, or to homozygous MR or PR subjects (see **Figure 5**). This finding evinces the long-term consequences of early rearing experiences on the serotonin system and that those consequences are made more or less extreme by experiential variation early in life. It is argued elsewhere that heritable influences on a variety of behaviors and developmental and psychopathological outcomes vary considerably across studies, indicating that genotypic effects are often dependent on the environment in which they are measured (Espinel and Higley, 2013). This replication punctuates this point, showing that CSF 5-HIAA concentrations during *Baseline* are lower in subjects with the *s* allele, but only if they are reared in the absence of mothers (PR).

While some studies with relatively small sample sizes indicate that the impact of early experience on the development of the monoamine systems persist beyond infancy (Higley et al., 1992, 1996), this study only included infant subjects. To investigate the long-term impact of early experience on the monoamine systems in later development, a longitudinal study including adult timepoints is of interest and would be an important contribution to the understanding of the persistence of the effects of early experience on monoamine functioning. Furthermore, it would be interesting to study the long-term predictive value of early experience-mediated monoamine functioning on behavioral and psychopathological outcomes.

Taken together, these findings indicate the importance of a sensitive caregiver early in life for normative neurodevelopment. These findings also suggest that stressful life events may elicit important differences in the monoamine metabolite systems, highlighting the importance of a caregiver that is sensitive to its infant's emotional needs, which is the experience that the infant's "experience-expectant" brain expects for normative development to occur. These findings are strengthened by the use of a rhesus monkey model, where the early rearing environment is randomly assigned and other extraneous variables can be closely controlled. Overall, these findings are an important step in understanding the impact of a caregiver that responds sensitively to its infant's psychological needs early in life, leading to normative overall neurobiological development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the NIH Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EW and JDH were responsible for study concept and design. MS, SL, CB, SS, and JDH contributed to the acquisition of the data. EW, NG, AS, and JDH assisted with analyses and interpretation of the findings. EW and JDH drafted the initial manuscript. EW, NG, JH, AS, MS, SL, CB, SS, and JDH critically reviewed content and approved the final version of the manuscript for publication. All authors contributed to the article 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/fnhum.2021.624676/full#supplementary-material

Supplementary Figure 1 | Representative Gel for 5-HTT Genotyping. Representative gel showing amplicons generated by PCR for the length variant in the regulatory region for the 5-HTT: going from left to right are the size standards and genotypes (*ss, Ls, LL, LL, ss, LL, Ls, LL, and LL*).

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Early Life Stress and the Fate of Kynurenine Pathway Metabolites

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Early life stress (ELS) precedes alterations to neuro-immune activation, which may mediate an increased risk for stress-related psychiatric disorders, potentially through alterations of central kynurenine pathway (KP) metabolites, the latter being relatively unexplored. We hypothesized that ELS in a non-human primate model would lead to a reduction of neuroprotective and increases of neurotoxic KP metabolites. Twelve adult female bonnet macaques reared under conditions of maternal variable foraging demand (VFD) were compared to 27 age- and weight-matched non-VFD-exposed female controls. Baseline behavioral observations of social affiliation were taken over a 12-week period followed by the first cerebrospinal fluid (CSF) sample. Subjects were then either exposed to a 12-week repeated separation paradigm (RSP) or assigned to a "no-RSP" condition followed by a second CSF. We used high-performance liquid chromatography for kynurenine (KYN), tryptophan, 5-hydroxyindoleacetic acid, kynurenic acid (KYNA), and anthranilic acid (ANTH) as a proxy for quinolinic acid determination. At baseline, social affiliation scores were reduced in VFD-reared versus control subjects. CSF log KYNA and log KYNA/KYN ratio were lower in VFD-reared versus control subjects. CSF log KYNA/KYN was positively correlated with CSF log ANTH in VFD only (r = 0.82). Controlling for log KYNA/KYN, log ANTH was elevated in VFD-reared subjects versus controls. CSF log KYNA/KYN obtained post-RSP was positively correlated with mean social affiliation scores during RSP, specifically in VFD. ELS is associated with a reduced neuroprotective and increased neurotoxic pathway products. That the two contrasting processes are paradoxically correlated following ELS suggests a cross-talk between two opposing KP enzymatic systems.

Keywords: kynurenine (KYN), Neuroprotection, kynurenic acid (KYNA), early life stress (ELS), Anthranilic acid (AA), non human primate, Major depressive disorders, childhood adversities

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INTRODUCTION

The role of pathogenic neuro-immune activation is increasingly recognized in serious psychiatric disorders such as major depression, bipolar disorder, and schizophrenia (Doorduin et al., 2009; Rao et al., 2010; Frick et al., 2013). During pro-inflammatory states, peripheral elevations of plasma cytokines, such as interleukin-1, interleukin-6, tumor necrosis factor-α, and interferon-γ

(IFN-γ), induce central expression of the enzyme indoleamine 2,3-dioxygenase (IDO). IDO diverts central tryptophan away from serotonin biosynthesis directly into the kynurenine pathway (KP). Two opposing neuroactive KP metabolites, kynurenic acid (KYNA), which is neuroprotective, and quinolinic acid (QUIN), which is neurotoxic, are now a primary focus in our understanding of the pathogenesis of mood and anxiety disorders and certain stress-related disorders (Savitz, 2020). At "the fork in the road," kynurenine is metabolized either to KYNA by kynurenine aminotransferases (KATs) or to 3-hydroxykynurenine (3-HK) by kynurenine-3-monooxygenase (KMO). The latter metabolic pathway leads to anthranilic acid (ANTH) and then to QUIN. Under basal conditions, most of kynurenine in the brain is metabolized to KYNA through KATs (Parrott and O'Connor, 2015). However, pro-inflammatory cytokines shift kynurenine metabolism through KMO to 3-HK and the neurotoxic pathway (Parrott et al., 2016a). Evidently, a preferential expression of either KATs or KMO may have major implications for the maintenance of affective state.

Childhood adversity may represent an independent, yet potentially preventable, risk factor for altered neuro-immune activation that persists into adulthood (Danese et al., 2007). Dysfunctional immune responsivity is cited as an important variable linking early life stress (ELS) and poor health outcomes (Frick et al., 2013). ELS is, therefore, a well-acknowledged risk factor for the development of a range of stress-related disorders that include psychological, psychosomatic, and physical conditions (Felitti and Anda, 2010). ELS has been associated with elevations of peripheral inflammatory markers in both humans (Carpenter et al., 2010) and non-human primate species (i.e., macaque) (McCormack et al., 2006). A consideration of alterations in neuroimmune signaling and KP metabolism has been primarily studied in rodents (Brenhouse et al., 2018; Allen et al., 2020).

Neuro-immune-mediated alterations of the KP catabolites have been of growing interest as a) acute KP activation may theoretically rapidly deplete tryptophan resources necessary for serotonin biosynthesis (Sublette and Postolache, 2012); b) via increases in quinolinic acid, there is an enhancement of N-methyl D-aspartate (NMDA)-mediated neurotoxicity (Brundin et al., 2016); and c) through KYNA decreases, there is a reduction of NMDA antagonist effects and subsequent compromise of regional brain neuroprotection (Kim and Won, 2017).

QUIN concentrations, the final metabolite of the neuroexcitatory arm of the KP, are elevated in the lumbar cerebrospinal fluid (CSF) of MDD patients who attempted suicide in comparison to patients with MDD without suicidality (Brundin et al., 2016). KYNA, by contrast, functions in a neuroprotective role, thereby comprising an alternate pathway for kynurenine being funneled into the neuroexcitatory pathway. KYNA demonstrates high affinity for the NMDA receptor, thereby preventing neuronal cell death in the face of glutamate excitotoxicity (Andiné et al., 1988; Leib et al., 1996). The approval of biologic agents that specifically suppress inflammation has been used quite extensively in clinical trials examining for antidepressant effects in treatment-resistant depression (TRD) and highlights a noteworthy advancement in psychopharmacology (Raison et al., 2013; Lee et al., 2020). Thus, the elucidation of the KP catabolites and brain function in psychiatric illness may enhance therapeutic opportunities, with the goal of pursuing incrementally more effective treatments that could be applied trans-diagnostically. Recent advances have elucidated a role for the immune system, which is now recognized to be an instrumental component to cause depression from neurotoxicity rather than tryptophan depletion (Wichers et al., 2005), and although modest tryptophan decreases have been associated with depression, these occur independent of IDO activity (Wichers et al., 2005).

ELS inevitably exerts epigenetic modifications that may exhibit persistent phenotypic effects across emotional development (Suderman et al., 2012). The configuration of these non-human primate epigenetic modifications may bear relevance toward the ability to maintain behavioral and physiological homeostasis in humans (McEwen, 1998, 2003; Howell and Sanchez, 2011; Hostinar and Gunnar, 2013). The novel concept of the "compensatory immune-regulatory reflex system" (CIRS) proposes that a substantial number of patients with bipolar disorder or major depression have aberrant immune-inflammatory response systems (Maes and Carvalho, 2018). Consistent with a proposed "immune gate of depression" model, early neuroimmunological development is thought to be formative to the underlying risk and development of depressive disorders (Kowalczyk et al., 2019). Non-human primate models of ELS therefore present a cogent model for providing an initial neuroimmune trigger and activation that could potentially facilitate understanding of the complex pathways to depression, anxiety disorders, and other stress-related disorders.

In this study, we seek to develop an understanding of psycho-neuro-immune development as pertains to kynurenine metabolism. The neurodevelopmental trajectory of KP metabolism and its modulation by early life experience are of clear translational significance. We hypothesized that ELS would be associated with significant alterations in key KP metabolites that suggest persistently elevated brain neurotoxicity and reduced neuroprotection. Further, we sought to demonstrate that these KP metabolite alterations would relate to behavioral profiles reflective of affective distress.

METHODS

Subjects

Twelve adult variable foraging demand (VFD)-reared female subjects were studied. In order to provide an age- and weight-matched control, 27 non-VFD reared subjects were studied as a comparison (see **Table 1**). As can be seen from **Table 1**, there were no age or weight differences between the VFD and the non-VFD control group. All procedures were performed in careful accordance with the Guide for the Care and Use of Laboratory Animals[1].

[1]https://www.aaalac.org/resources/theguide.cfm

TABLE 1 | Means and standard errors for ages and weights of non-human primate groups.

Rearing	*N*	Age (mean ± SE) (years)	Mass (mean ± SE) (kg)
VFD	12	14.77 ± 1.07[a]	5.80 ± 0.38[b]
Non-VFD	27	16.18 ± 0.73[a]	5.37 ± 0.26[b]

[a]$F_{1,37}$ = 1.16, p = 0.28; [b]$F_{1,37}$ = 0.84, p = 0.36. VFD, variable foraging demand; SE, standard error.

Rearing Methods

For the VFD-rearing exposure, as previously described (Coplan et al., 1996), mothers were confronted with an environment in which adequate amounts of food were available throughout but in which the amount of time and effort necessary to obtain daily rations was unpredictable (Rosenblum and Paully, 1984). The VFD maternal food procurement schedule consisted of eight 2-week blocks in which food was either readily found (low foraging demand—LFD) alternating with 2-week blocks in which food procurement entailed more time, complexity, and effort (high foraging demand—HFD). A total of four consecutive epochs were implemented—each epoch comprised a 2-week period of LFD alternated with a two-week period of HFD—which in total comprised the 16-week maternal VFD exposure experimental period. For purposes of uniformity, the introductory 2-week block was always designated as an LFD epoch. Infant age at the time of onset of the VFD procedure was approximately 3 months of age. To "vary" maternal foraging demand, a simple device, referred to as "the foraging cart," is implemented. Mothers forage for food that can either be buried in wood chip (HFD) or left freely exposed in containers within the cart (LFD). Mothers are required to manually search for and retrieve the apportioned food by reaching through multiple apertures situated on the side of the cart. Further details of the foraging cart and the VFD procedure itself may be found in Andrews and Rosenblum (Andrews and Rosenblum, 1991).

Observations of Adult Social Affiliation

Prior to initiation of adult observation measurements, VFD subjects lived in three social groups consisting of four subjects each (n = 12) that had been stabilized for at least 2 months (see flow chart on **Figure 1**). Details of non-VFD housing arrangements are provided in the **Appendix**. For each

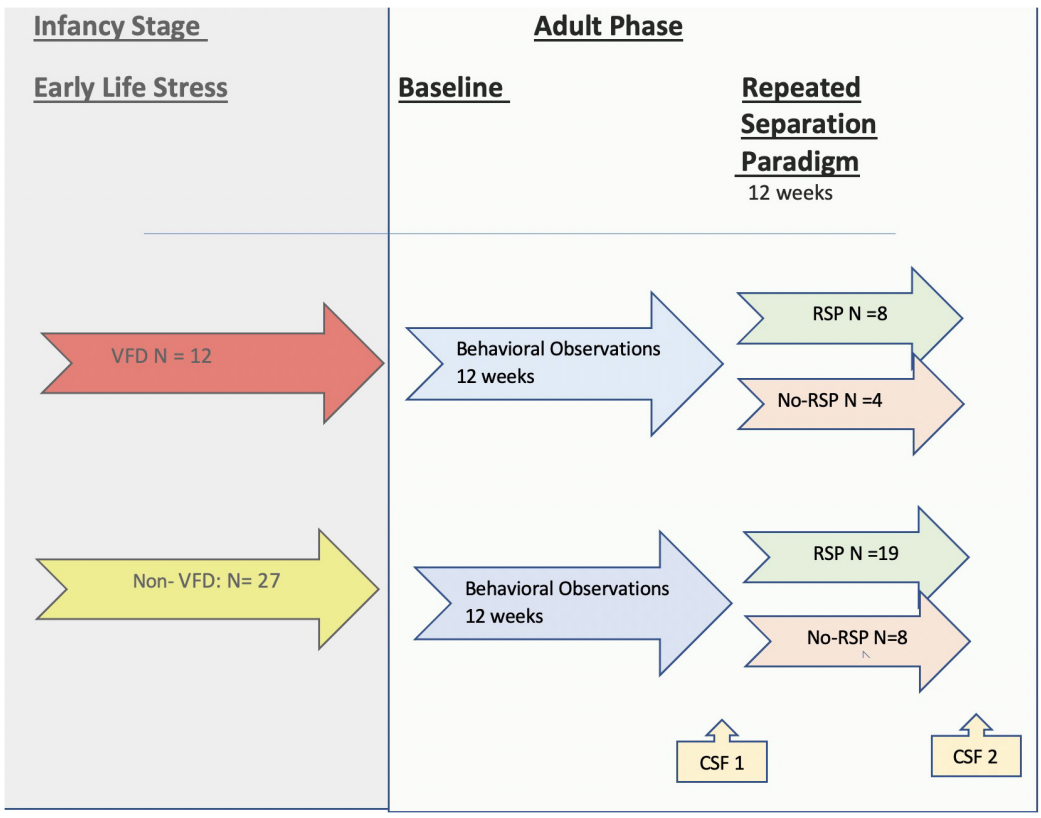

FIGURE 1 | Flow chart for timeline of the current non-human primate study. A flow chart is provided depicting the two phases of the study. In the left-hand panel (in gray) is the infancy stage, during which the early life stress exposure occurs—the maternal variable foraging demand (VFD) paradigm (red arrow)—or the control condition—the non-VFD group (yellow arrow) where VFD is not imposed. In the right-hand panel side (in green) is the adult phase of the study. The baseline period comprises 12 weeks of blinded behavioral observations (blue arrows), whereas in the ensuing 12-week period, a portion of both groups are exposed to the repeated separation paradigm (RSP) (light green) or to no-RSP (light red). The number of subjects at each phase is shown in the diagram. The first cerebrospinal fluid (CSF) sample was obtained following the baseline observation period, whereas the second CSF was obtained following the RSP period (yellow arrows at bottom). Abbreviations: VFD, variable foraging demand; RSP, repeated separation paradigm; CSF, cerebrospinal fluid.

experimental condition, every subject within a given social pen received the same treatment. VFD and non-VFD subjects were always housed in separate pens.

Baseline behavioral observations were conducted over a 12-week period (data on 6 weeks are available) that preceded exposure or no-exposure to a repeated separation paradigm (RSP) protocol of 12 weeks (data are available on 10 weeks), during which behavioral observations identical to the baseline period were conducted. All observation sessions were conducted at the same general time of day (from 10 am to 1 pm) to control for any potential diurnal rhythms in social behavior. The behavioral ratings were conducted by a trained technician (inter-rater reliability $\lambda > 0.90$), blinded to the rearing group. For 20 successive 30-s observation cycles, the presence or absence of social affiliative behaviors was identified for each animal within the social group. Thirty-second intervals were digitally timed using a tone delivered by headphones so that the tone could only be heard by the observer. Instances of hugging, grooming, receiving grooming, passive contact, and proximity were tallied and later summed to yield an overall affiliation score for each session. For the majority of subjects, a full 36 behavioral observation sessions were completed over a 12-week baseline period, generally on Mondays, Wednesdays, and Fridays (6 weeks of behavioral observations were tallied). A similar number of behavioral observations were available for the RSP (five 2-week blocks were tallied). The data is presented as the mean score/session collapsed over a 2-week block.

Repeated Separation Paradigm

The adult RSP protocol utilized in the current study was based on a protocol developed by Higley et al. (1996) (Erickson et al., 2005). Please see the flow diagram for subjects from each rearing group that received RSP. The duration of the protocol lasted for a total of 12 weeks, during which time animals that were housed in social groups in group pens were placed in individual cages for 2.5 days (Friday evening to Monday morning) each week, leading to repeated separations and reunions. During the separation period, animals were housed in individual cages that were covered with black polyethylene covering that blocked direct visualization of other animals but not the auditory communications or olfactory cues produced by closely housed individual animals. Then, for the remainder of the week, animals were placed back into their usual social environment in the home pen. The control "no RSP" condition was non-exposed to repeated separation. All subjects housed within a given pen were either all exposed to RSP or were in the "no RSP" control condition. For VFD, four subjects in one pen underwent RSP, whereas two pens of four subjects each underwent no-RSP. Of note, there was no instance where VFD subjects were housed with non-VFD subjects. All protocols were done in compliance with the Institutional Animal Care and Use Policy of The State University of New York Downstate Health Sciences University/State University Hospital.

CSF Methods

Subjects were taken from their home cage and placed in carrying cages, a routine procedure for cleaning purposes. For CSF sampling, subjects were released into restraint cages and intramuscular ketamine (15 mg/kg) was administered. Sedation of subjects was achieved in less than 5 min after exiting the carrying cage. Cisternal CSF sampling was performed immediately following sedation. CSF samples were then placed in Gant tubes and stored in a −70°C freezer. As part of the IACUC protocol, there is an option of administering analgesics in the event any of the animal subjects were experiencing pain or discomfort following the CSF draw. The procedure has been very well tolerated, and the subjects do not appear to experience pain or discomfort even when observed after the anesthetic and analgesic effects of ketamine have worn off.

Kynurenine Pathway Metabolites

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze the extraction of CSF samples, using the method described by the Clinical Laboratory and Standards Institute (Clarke et al., 2014). The samples were used to measure concentrations of CNS metabolites pertaining to tryptophan metabolism (TRP), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KYNA), and anthranilic acid (ANTH). Linear ranges were determined to assure that the methodology used would provide accurate quantitation and reproducibility of the assay. See supplement for comprehensive description of methodology implemented. Data from our own and other laboratories indicate relative stability of monoamine metabolite concentrations (Higley et al., 1993, 1996; Kaplan et al., 1994; Shively, 1998). All laboratory personnel conducting the biochemical assays were blind to the subjects' rearing status.

Statistical Methods

Age and weight of animals were matched and not used as covariates. Statistica 12.0 by StatSoft^{TM} was used for analysis of all data. Outliers, which were defined as data points three deviations outside a mean that included both rearing groups, were to be excluded from the analyses. *T*-tests were performed to test for baseline characteristics. All kynurenine metabolites and their ratios were log-transformed to protect against non-homogeneity of variance. Non-parametric testing was used when appropriate. Generalized linear models were used to test for VFD rearing effects when using covariates. Factorial ANOVAs were used in the general liner model (GLM), generating an interactive term included in the overall model, specifically when comparing correlations between rearing groups. Significance was set at $p \leq 0.05$, two-tailed.

RESULTS

Age and Weight

The two groups were closely matched for age and weight and were not significantly different from each other (**Table 1**).

Affiliation Scores at Baseline

Affiliation scores combined the last two of the three 2-week baseline periods and used the first baseline period as covariate. There was an overall effect for group (VFD mean ± SD:

64.67 ± 6.86, $N = 12$ vs non-VFD mean ± SD: 85.34 ± 4.75, $N = 25$; $F_{1,34} = 6.01$, $p = 0.019$).

CSF Log Kynurenic Acid at Baseline

Log KYNA was significantly lower in VFD-reared subjects versus non-VFD controls at baseline (mean ± SD: 2.05 ± 0.61, $N = 10$ vs 3.32 ± 1.2, $N = 15$; t-value$_{23} = 2.86$, $p = 0.009$; Kruskal–Wallis $H_{1,25} = 6.23$, $p = 0.012$). There were no significant group effects for logged CSF tryptophan, kynurenine 5-HIAA, or anthranilic acid. Data related to ratios are presented below.

CSF Log Kynurenic Acid/Kynurenine Ratio at Baseline

CSF log KYNA/KYN ratio in VFD subjects was lower in comparison to non-VFD controls (mean ± SD: 2.00 ± 0.88, $N = 10$ versus 3.07 ± 1.21, $N = 14$; t-value$_{22} = 2.34$, $p = 0.03$) (see **Figure 2** for non-parametric Kruskal–Wallis confirmation).

Baseline CSF Kynurenine Pathway Metabolites of the Neurotoxic Arm

We utilized a GLM factorial ANOVA using CSF log ANTH as the dependent variable, CSF log KYNA/KYN as a continuous predictor variable, and rearing group as the categorical variable.

The goal was to assess the relationship between the KP neurotoxic and neuroprotective arms, taking into account that CSF log KYNA/KYN was indeed reduced in VFD at baseline. The model therefore consisted of the following variables as predictors of log ANTH: VFD grouping, log KYN/KYNA$_{ratio}$, and the interactive term VFD*log KYN/KYNA ratio. There was an overall VFD effect ($F_{1,19} = 6.97$; $p = 0.016$), indicating that when controlling for log KYNA/KYN ratio, VFD exhibited elevations of log ANTH [mean (95% confidence interval, CI): −0.44, (−0.88 to −0.01); $N = 9$] in comparison to non-VFD [mean (95% CI): −0.72 (−1.07 to −0.37); $N = 14$]. There was a significant effect for log KYNA/KYN ratio prediction of log ANTH ($F_{1,19} = 10.47$; $p = 0.004$) in a positive direction, when rearing groups are combined. There was, however, a marked VFD*log KYNA/KYN interactive effect ($F_{1,19} = 13.91$; $p = 0.0014$) reflective of a positive correlation between log KYNA/KYN and log ANTH in VFD ($r = 0.82$, $p = 0.007$; $N = 9$) in comparison to non-VFD-reared controls ($r = -0.14$; $p = 0.61$, $N = 14$) (**Figure 3**). To put the latter interactive effect into context, an effect size was assessed by partial η^2 of 0.42 (Cohen, 1973), where a large effect size is deemed 0.14, which places the current effect size at three-fold greater than a large effect size.

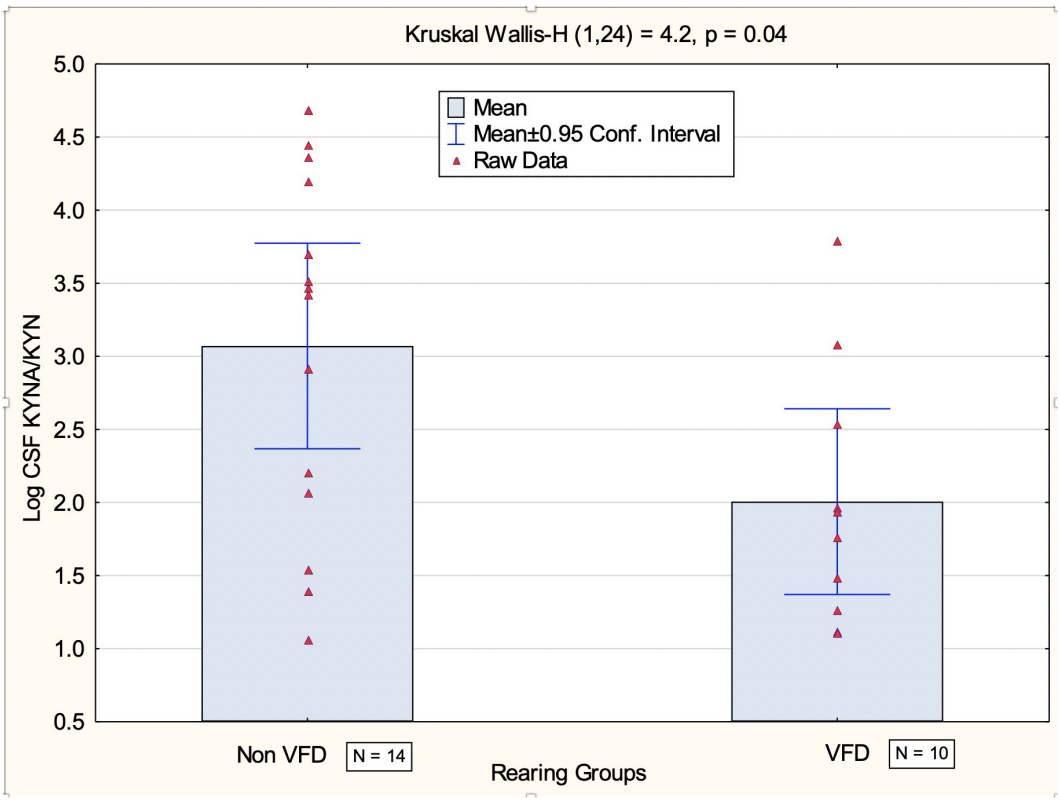

FIGURE 2 | Comparison of CSF log kynurenic acid/kynurenine ratio in VFD-reared macaques and non-VFD subjects at baseline. Significant reductions were observed at baseline using the non-parametric Kruskal–Wallis test in VFD CSF log kynurenic acid/kynurenine ratio ($N = 10$) in comparison to non-VFD-exposed controls ($N = 14$). The data provide evidence for reduction of the neuroprotective arm of the kynurenine pathway following VFD rearing as kynurenic acid functions as an NMDA antagonist. Abbreviations: VFD, variable foraging demand; CSF, cerebrospinal fluid; NMDA, N-methyl D-aspartate.

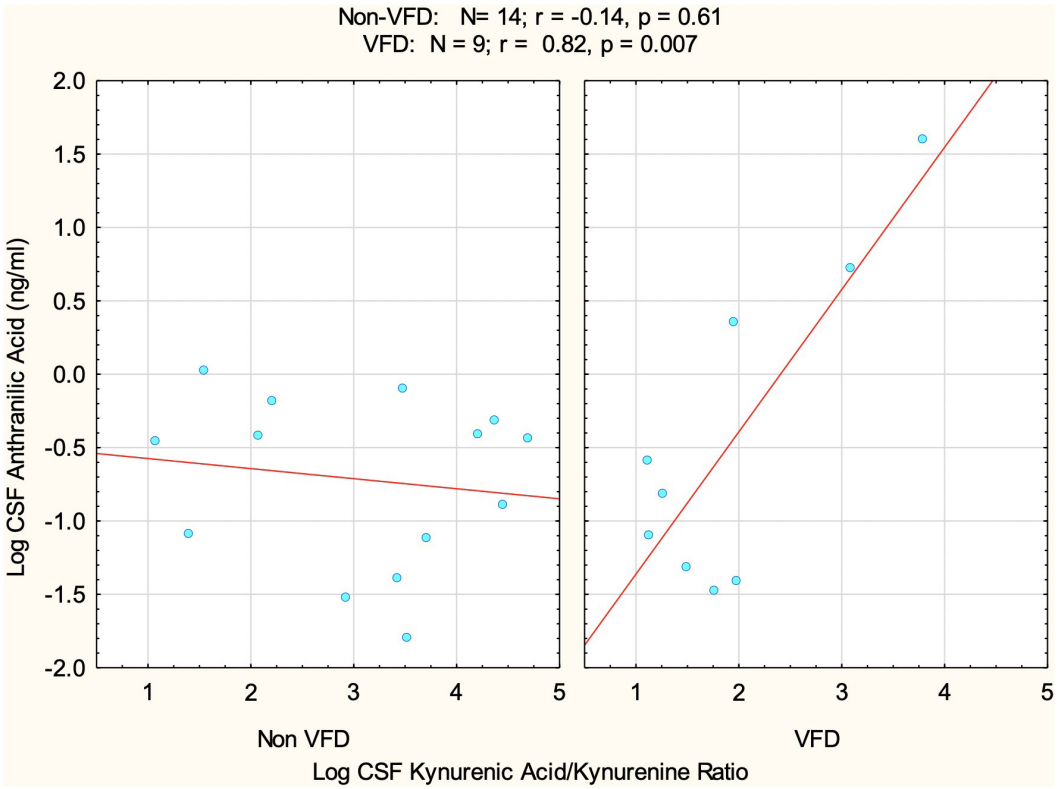

FIGURE 3 | The relationship between baseline CSF log kynurenic acid/kynurenine ratio (KYNA/KYN) and CSF log anthranilic acid (ANTH) as a function of early adversity. A positive correlation was observed at baseline between the CSF log KYNA/KYN and CSF log ANTH, specifically in VFD subjects ($N = 9$) in comparison to the non-correlation in normatively reared controls ($N = 14$). There was a significant VFD*KYNA/KYN interactive effect ($F_{1,19} = 13.91$; $p = 0.0014$) rendering an effect size (partial $\eta^2 = 0.42$) that is three-fold greater than a large effect size, deemed as 0.14. The data suggest a close synchronization of the neuroprotective and neuroxic arms—CSF ANTH serves as a proxy for the latter—within the kynurenine pathway following the VFD form of early life stress. Abbreviations: VFD, variable foraging demand; KYNA, kynurenic acid; KYN, kynurenine; CSF, cerebrospinal fluid; ANTH, anthranilic acid.

CSF Log Kynurenic Acid/Kynurenine Ratio in Response to Repeated Separation Paradigm

A factorial ANOVA was performed including the following variables: CSF log KYN/KYN as the independent measure and VFD grouping, RSP exposure, and a VFD*RSP interactive effect (**Appendix Table 1**). It was not possible to integrate baseline CSF log KYNA/KYN ratio data into a repeated measure design using GLM, as values for non-VFD no-RSP exposure were not available (see **Appendix** for missing KP metabolite values). An overall VFD effect was observed ($F_{1,30} = 16.73$, $p = 0.0003$) consistent with the view that CSF log KYNA/KYN ratio is reduced in VFD irrespective of whether subjects were exposed to the RSP or not. Although no overall effect of the RSP on CSF log KYNA/KYN was evident ($F_{1,30} = 0.22$, $p = 0.64$), a significant effect of VFD*RSP interactive effect for CSF log KYNA/KYN was observed ($F_{1,30} = 12.12$, $p = 0.0016$) (see **Figure 4**). Non-VFD subjects exhibit decreases in CSF log KYNA/KYN ratio post-RSP in comparison to no RSP exposure [mean (95% CI): no RSP: 4.08 (3.26 to 4.89), $N = 5$ versus RSP: 3.01 (2.57 to 3.45), $N = 17$; *post hoc* least square difference (LSD); $p < 0.025$]. By comparison, VFD subjects exposed to RSP exhibit increases in CSF log KYNA/KYN in comparison to VFD with no RSP exposure [mean (95% CI): no RSP: 1.39 (0.75 to 2.03); $N = 8$ versus RSP: 2.79 (1.88 to 3.70); $N = 4$; *post hoc* LSD; $p = 0.04$]. CSF log KYNA/KYN ratio of VFD subjects exposed to RSP was not distinguishable from non-VFD exposed to RSP (see **Figure 4**). A scatterplot with error bars using 95% CIs of CSF log KYNA/KYN ratios is presented in the **Appendix** to examine a comparison of values obtained from the first to the second CSF tap (see **Appendix Figure 1** and flow chart in **Figure 1**).

Relationship of CSF Log Kynurenic Acid/Kynurenine Ratio to Social Affiliative Behavior After Repeated Separation Paradigm as a Function of Variable Foraging Demand Rearing

In order to control for the effects of RSP in relation to CSF log KYNA/KYN ratio and affiliative behavior, we entered the following variables into the GLM: VFD grouping, VFD*RSP interactive term, CSF log KYNA/KYN taken from the second CSF tap (see flow chart in **Figure 1**), and the VFD*CSF log KYNA/KYN interactive term. The five scores of social affiliative

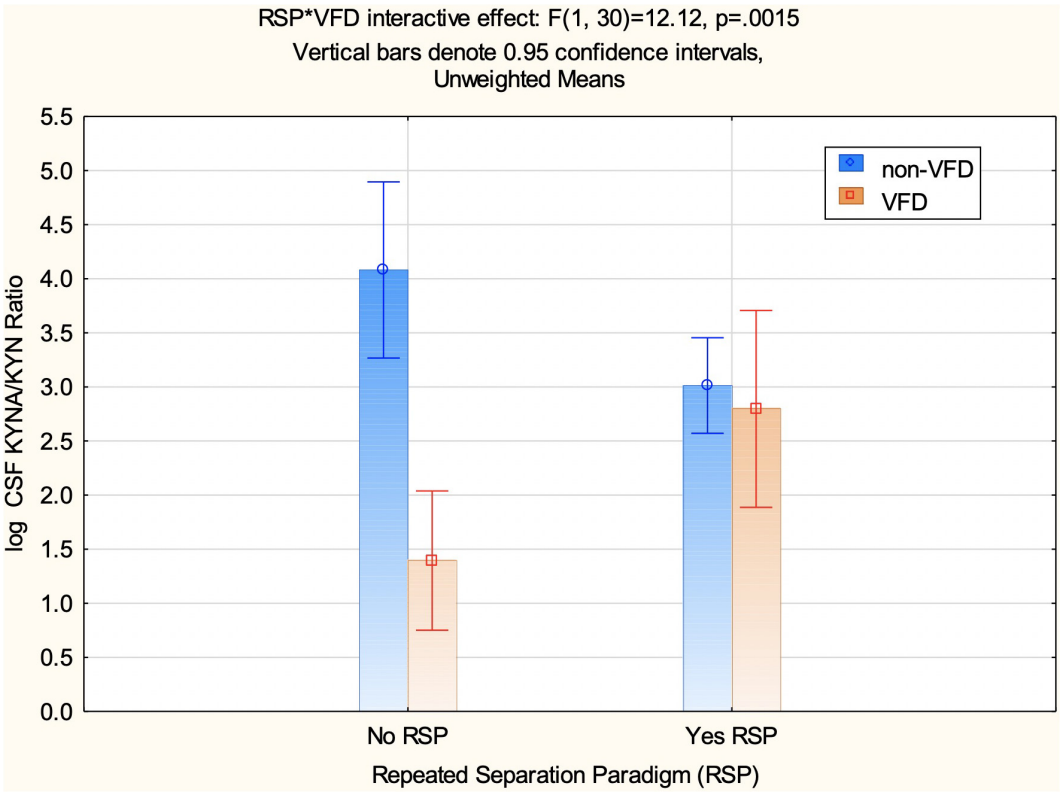

FIGURE 4 | CSF log kunurenic acid/kynurenine ratio in response to the repeated separation paradigm as a function of variable foraging demand exposure during early life. There is a repeated separation paradigm (RSP)*VFD interactive effect for CSF log KYNA/KYN obtained from the CSF following the RSP phase ($F_{1,30}$ = 12.12; p = 0.0016) (see **Figure 1**). CSF log KYNA/KYN is lower in non-VFD subjects who were exposed to the RSP (N = 17) in comparison to those who did not (N = 5; p = 0.0001), whereas VFD exposed to the RSP (N = 8) exhibited significantly increased CSF log KYNA/KYN ratios in comparison to no-RSP-exposed VFD subjects (N = 4; p = 0.04). Non-VFD unexposed to RSP exhibited exhibited greater CSF log KYNA/KYN in comparson to VFD unexposed to RSP (p = 0.00001). By contrast, post-exposure to RSP, VFD and non-VFD exposed are not distinguishable (p = 0.66). Abbreviations: VFD, variable foraging demand; KYNA, kynurenic acid; KYN, kynurenine; CSF, cerebrospinal fluid; RSP, repeated separation paradigm.

behavior observed during the RSP exposure period served as the repeated measure. CSF log KYNA/KYN ratio positively predicted social affiliative behavior, measured over five separate 2-week blocks ($F_{1,28}$ = 13.05, p = 0.001). However, only in the VFD subjects did CSF log KYNA/KYN ratio positively predict social affiliative behavior, whereas in non-VFD, this relationship was not evident (VFD*log KYNA/KYN interactive effect; $F_{1,28}$ = 10.21; p = 0.0034). For example, in **Figure 5**, of the five 2-week blocks, the second block shows a positive relationship in VFD between log KYNA/KYN and social affiliation (r = 0.82, N = 12, p = 0.007) in comparison to the corresponding correlation in the non-VFD subjects (r = −0.14, N = 22, p = 0.61). = A repeated-measures*VFD*log KYNA/KYN three-way interactive effect ($F_{4,112}$ = 5.79; p = 0.0003) was observed (see **Appendix Table 1**). Univariate analyses confirmed that three of the five separate 2-week blocks each revealed VFD*log KYNA/KYN two-way interactive effects while controlling for RSP exposure*VFD interactive effects, indicating that the relationship showed some variability between blocks. The GLM for the second block renders a partial η^2 effect size of 0.50, at least 3.5-fold larger than a large effect size. In VFD, incremental increases in the CSF log KYNA/KYN ratio were associated with a commensurate increase in social affiliative behavior (see **Figure 5**). The mean of all five 2-week social affiliation tallies obtained during the repeated separation paradigm exposure period and CSF log KYNA/KYN correlate in VFD (r = 0.66, N = 12, p = 0.019).

It remained unclear whether the post-RSP CSF log KYNA/KYN correlation to social affiliative behaviors was indeed dependent on exposure to the repeated separation paradigm as only four of the 12 VFD subjects were in fact exposed to the RSP. We therefore conducted a GLM in which we used as the repeated measure the mean of the baseline three 2-week blocks and then the mean of the five RSP social affiliative behavior 2-week block tallies, categorical variable was whether there was exposure to the RSP paradigm, and CSF log KYNA/KYN derived from the second CSF draw served as the continuous variable. In addition, we used a factorial design that generated a three-way interactive term of repeated measures*CSF log KYNA/KYN*repeated separation paradigm. This term was in fact significant ($F_{1,8}$ = 8.70, p = 0.018). *Post hoc* Pearson's correlations revealed that there was indeed a significant positive relationship between CSF log KYNA/KYN and mean

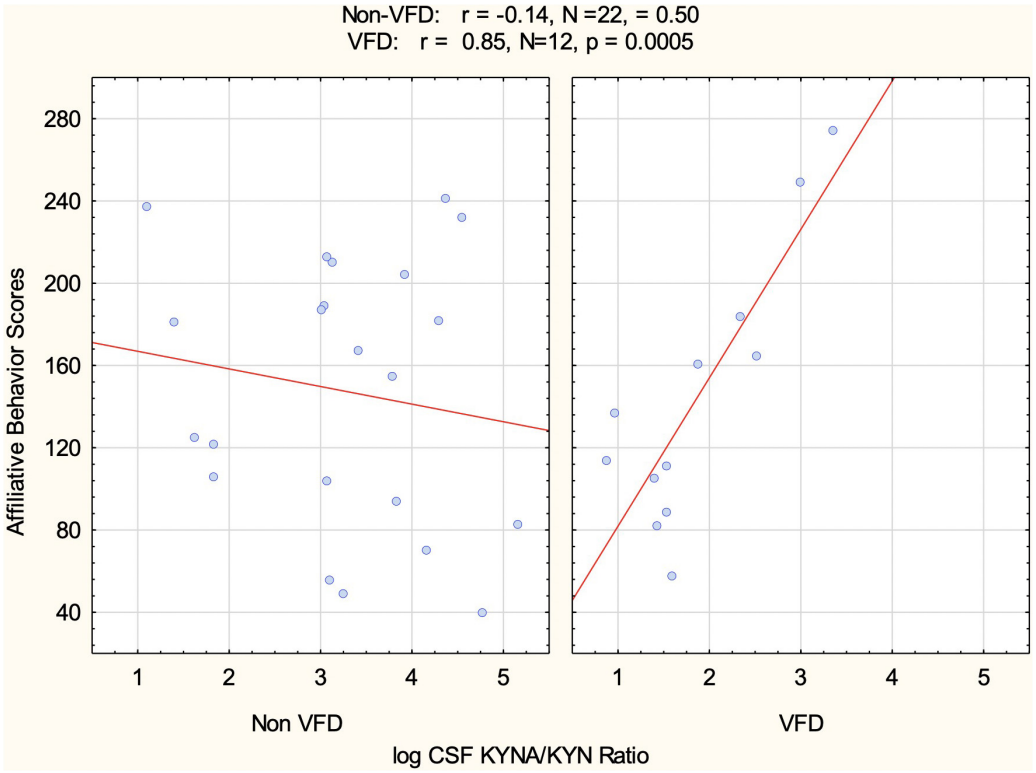

FIGURE 5 | CSF log kynurenic acid/kynurenine ratio correlation with social affiliation observed during repeated separation paradigm as a function of the variable foraging demand form of early life stress. Only in VFD was there a positive relationship between social affiliation cores and the neuroprotective index represented by CSF KYNA/KYN. The positive relationship was in the expected direction. For any increment in VFD CSF log KYNA/KYN, there was a commensurate increase in social affiliation. Of note, this was the affiliation behavioral scores of the second of the five 2-week behavioral blocks (see *RESULTS* for further information). Abbreviations: VFD, variable foraging demand; KYNA, kynurenic acid; KYN, kynurenine; CSF, cerebrospinal fluid.

social affiliation scores for the RSP-exposed subjects (r = 0.98, N = 4, p = 0.017) versus RSP non-exposed subjects (r = 0.14, N = 8, p = 0.74), supporting the view that the effects were due to the RSP-exposed subjects. There was no significant relationship between the mean baseline scores of social affiliation and CSF log KYNA/KYN derived from the second draw (r = 0.36, N = 4, p = 0.63), indicating that this relationship in RSP-exposed subjects was not due to a pre-existing relationship evident prior to RSP exposure.

The social affiliative behavioral response to the repeated separation paradigm is covered in **Appendix Figure 2**.

DISCUSSION

The findings in the current study corroborate our primary hypothesis that early life stress imparts persistent and pervasive changes to the neuro-immune landscape via alterations of KP metabolites. The findings include a reduction of social affiliative behaviors in VFD-reared versus non-VFD control subjects, confirming behavioral alterations in VFD versus non-VFD subjects during baseline home-cage observations (Rosenblum et al., 2001). Reduced baseline CSF log KYNA and lower CSF log KYNA/KYN ratio in VFD-reared subjects in comparison to non-VFD controls is suggestive that early life adversity associates with a relative reduction of neuroprotection alongside increases in KP metabolites within the neurotoxic arm. The aforementioned configuration of the KP metabolites was uniquely evident in VFD-reared subjects. That log KYNA is lower in VFD even when KYN is not indicates reduced activity of key enzymes such as kynurenine aminotransferases (KATs)—which convert KYN to KYNA (Okuno et al., 1991; Parrott et al., 2016b)—specifically in VFD-reared subjects. When controlling for log KYNA/KYN, there were elevations of CSF log anthranilic acid (ANTH) in VFD-reared subjects in comparison to non-VFD controls. The latter result implies an increased activity of KMO in relationship to KAT (Parrott and O'Connor, 2015; Parrott et al., 2016b), which converts kynurenine to 3-hydroxykynurenine and is then converted to 3-hydroxyanthranilic acid by kynureninase. The latter is converted to quinolinic acid by 3-hydroxyanthranilic acid dioxygenase (Krause et al., 2011). An unexpected positive correlation between CSF log KYNA/KYN and CSF log ANTH (Pearson's r = 0.82) is present—uniquely evident in VFD-reared subjects—and is suggestive of a synchronized activity between the neurotoxic and neuroprotective arms of the KP following ELS. The effect size of the VFD-specific synchronization exceeds a large effect size by a factor of three (Cohen, 1973). One plausible explanation that may

account for the inordinately high positive correlation between neuroprotective and neurotoxic arms observed in the VFD-reared subjects is a possible coordinated "cross-talk" between the KAT and KMO enzymatic activity.

Following 12 weeks of exposure as adults to the RSP, there are, when compared to the non-VFD no-RSP control group, reductions of CSF log KYNA/KYN obtained from the second blood draw, in RSP-exposed non-VFD. By contrast, for VFD-reared subjects, relative to the no-RSP control condition, CSF log KYNA/KYN is elevated in the RSP condition. The latter two RSP effects erase any non-VFD/VFD effects for CSF log KYNA/KYN post-RSP. Controlling for VFD*RSP effects, behavioral observations of social affiliation scored during the RSP paradigm period show a pattern of correlating positively with CSF log KYNA/KYN obtained following RSP, an effect specifically evident in VFD-reared subjects, but not in non-VFD subjects. Three of the five 2-week blocks of behavioral observations exhibited significant VFD-rearing*log KYNA/KYN interactive effects, indicating, for instance, a Pearson's $r = 0.82$, specifically in VFD, between social affiliation scores obtained from the second of the five 2-week blocks and CSF log KYNA/KYN. The effect size of the VFD rearing*log KYNA/KYN interactive variable in the prediction of total social affiliation scores is three-fold greater than what is considered a large effect size (Cohen, 1973). Further analyses in VFD reveal that CSF log KYNA/KYN, an index of neuroprotection dependent on the activity of the enzyme KAT, appears intimately related to the social affiliation scores specifically in the four subjects exposed to RSP. The eight no-RSP-exposed VFD subjects neither exhibited a significant correlation nor do the mean social affiliation scores observed during the baseline period prior in the four RSP-exposed VFD subjects.

ELS has been viewed as an independent and preventable risk factor for the elaboration of central hyper-immunity that persists into adulthood (Danese and Baldwin, 2017). This is the first report, to our knowledge, examining central kynurenine pathway (KP) metabolites in non-human primates following the VFD form of early life stress, although the absence of markers of inflammation or central activation of specific cytokines allows for only the suggestion of a persistent hyperimmune state.

Interestingly the effects on KP metabolism in VFD-reared non-human primates did not appear to impact serotonin biosynthesis, which would be expected to occur following extensive and rapid tryptophan diversion to the KP pathway via increased IDO enzymatic activity (Sublette and Postolache, 2012). In this study, both CSF 5-HIAA and CSF tryptophan at baseline did not appear to differ between rearing groups. Previous reports in three VFD-reared cohorts, in fact, have documented high CSF 5-HIAA in VFD-reared subjects, diminishing the likelihood of chronic central tryptophan depletion by high IDO activity (Coplan et al., 2014). The activity of IDO does not appear overtly hyperactivated in VFD as CSF kynurenine concentrations did not differ between the groups. Therefore, the view that early life stress persistently depletes serotonin availability through diversion of tryptophan to the kynurenine pathways and away from serotonin biosynthesis is not supported (Capuron et al., 2002; Capuron et al., 2003). Activation of the KP without an impact on serotonin metabolism is consistent with several related findings in major depressive disorders (Savitz et al., 2015). Future studies may be able to substantiate whether these alterations are a byproduct of low-grade inflammation that may confer vulnerability to depressive disorders and other stress-related disorders (Savitz et al., 2015).

Supporting the view that, following early life stress, CSF log KYNA/KYN ratio is highly relevant to the affective state of macaque subjects, we were able to demonstrate a significant coupling between a compromised neuroprotective profile and social affiliation, an effect specifically observed following the repeated separation paradigm. A separate study aimed at leveraging KMO inhibition is needed to more directly support the social effects of KYNA increases under conditions of chronic stress (Cohen, 1973). That the effect size of the relationship between KYNA/KYN and social affiliation, at its most dramatic, is three-fold greater than a "large effect size" is remarkable as further analyses reveal that this effect is attributable to four RSP-exposed VFD subjects only.

KYNA is produced in astrocytes by KAT and is generally thought to play a neuroprotective role as an allosteric modulator of the glycine site at the NMDA receptor (Guillemin, 2012; Dantzer and Walker, 2014; Schwarcz, 2016). Inhibition of glutamatergic neurotransmission through binding of KYNA to the glycine site of the NMDA receptor was found to decrease striatal dopamine release, with potential consequences for motivational states implicit to social affiliation (Wu et al., 2007). KMO knockout mice maintain the ability to produce KYNA but not QUIN. The KMO knockout prevents accumulation of lipopolysaccharide-induced KP neurotoxic metabolites in the dorsal hippocampus and emergence of depression-like behaviors (Parrott et al., 2016b). The large effect size, seen in the current study, for the two-way interactive effect of VFD*log KYNA/KYN in the prediction of affiliative behavior supports the notion that KP metabolites are sub-serving divergent central functions in ELS versus non-ELS subjects.

The repeated separation paradigm obscures VFD/non-VFD group differences evident in the no-RSP control condition. This effect is true for CSF log KYNA/KYN (see **Appendix Figure 1**) and social affiliation scores (see **Appendix Figure 2**), supporting the view that the RSP had significant yet opposing effects on VFD versus non-VFD subjects. Of note, neither the CSF log KYNA/KYN measured after the repeated separation paradigm predict baseline social affiliative behavior nor does baseline KYNA/KYN predict social affiliation during the repeated separation paradigm (data available on request). Thus, the repeated separation paradigm specifically precipitates the emergence of the relationship between KYNA/KYN and social affiliation.

The findings of the current study are consistent with several core domains as defined in the Research Domain Criteria (RDoC) matrix. The current study leverages a paradigm of ELS (in the form of maternal VFD) (Kaufman et al., 2015) and an adult experimental stress paradigm that utilizes aspects of the domain of social cognition (affiliation and attachment construct) (Casey et al., 2014). Finally, as a primary behavioral outcome, social affiliation and its counterpart, social withdrawal,

have also been conceptualized within the RDoC framework (Cuthbert, 2019). Social competence has been viewed to depend on functional integrity of three of the RDoC domains: negative valence systems (for example, fear and anxiety in initiating or causing social contact), positive valence systems (for example, reward in response to prosocial behavior), and cognitive systems (Karhson et al., 2016). These convergent domains within the RDoC constructs may enhance replicability and translational value.

Limitations of the study include the examination of only female subjects. It is of note that human plasma KYNA levels are reduced in females and particularly women on oral contraceptives (Meier et al., 2018). It is unclear if these human sex differences translate to macaques. Nevertheless, there is a risk of generating type II errors through inclusion of both sexes when the total number of study subjects is small. Even substantial behavioral and biological differences may not be detected when including sex as a factor because of the small number of subjects within each cell. Nevertheless, adequately powered studies including both sexes are warranted. Because of technical difficulties, we were unable to determine CSF concentrations of QUIN. We were, however, able to measure ANTH, a metabolite of the neurotoxic branch of the KP and a direct, obligate precursor molecule to QUIN. ANTH is converted to QUIN by 3-hydroxyanthranilic acid dioxygenase, an enzyme that does not appear to be influenced by neuroinflammation (Krause et al., 2011). In addition, missing values for various KP metabolites may have led to uneven numbers in the analyses but have nevertheless sufficed to evince highly significant results. The translational significance of reduced social affiliative behavior, at baseline, as an indicator of affective distress in the bonnet macaque may have been inadequately clarified. The repeated separation paradigm, in fact, reduces social affiliation in non-VFD-reared subjects of sufficient magnitude to be rendered indistinguishable from VFD subjects. The reductions of social affiliation in non-VFD exposed to RSP support the view that even contemporaneous adult stress affects patterns of social affiliation.

In summary, ELS is associated with persistent reductions in KP metabolites integral to the neuroprotective arm, such as KYNA, and increases of KP metabolites within the neuroprotective arm, such as ANTH. The collective findings suggest that developmental alterations in neuro-immune activity may extend to the KP in adulthood and may be behaviorally evident in changes of social affiliative behavior. Of note, the underactive neuroprotective arm and overactive neurotoxic arm evident in the current study are paradoxically *positively* correlated specifically in VFD subjects. That these two opposing systems are positively correlated specifically in VFD implies persistent and coordinated cross-talk between the KAT and KMO enzyme systems. Only in VFD does exposure to the repeated separation paradigm reveal a novel relationship between social affiliation and kynurenine metabolites serving neuroprotection. Future studies are needed to establish the presence of perturbed inflammatory status in conjunction with KP metabolite alterations —a model which may serve as an invaluable opportunity for evaluation and development of novel therapeutic options.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by SUNY Downstate, Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SAS and JDC drafted manuscript, interpreted data, and critically revised manuscript through submission and acceptance. JDC completed analysis of data. RG, AR, JT, SF, and TP assisted with acquisition of data and made significant contributions to finalizing of 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/fnhum.2021.636144/full#supplementary-material

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Adult Women First Exposed to Early Adversity After 8 Years Old Show Attentional Bias to Threat

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Exposure to early adversity (EA) is associated with long-lasting dysregulations in cognitive processes sustained by brain regions that are sensitive to stress hormones: the hippocampus, the amygdala, and the prefrontal cortex. The Life Cycle Model of Stress highlights the importance of considering the timing at which EA began, as these brain regions follow distinct developmental trajectories. We aimed to test this hypothesis by assessing whether adults exposed to EA exhibit different cognitive patterns as a function of the age at which they were first exposed to EA. Eighty-five healthy men and women aged 21–40 years old (y/o) exposed to EA, as assessed by the Adverse Childhood Experience Questionnaire, were grouped based on the age of first exposure to EA: 0–2 y/o ("Infancy": hippocampal development), 3–7 y/o ("Early childhood": amygdala development) and after the age of 8 ("Childhood/Adolescence": frontoamygdala connectivity development). Declarative memory, attentional bias to threat and emotion regulation were measured. Results revealed increased attentional bias to threat in women first exposed to EA after 8 years. This result is in line with the Life Cycle Model of Stress and highlights the importance of considering the age at exposure to EA when investigating the effects of EA on cognitive processes.

Keywords: early adversity, minimal age at exposure, attentional biases, emotion regulation, declarative memory

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INTRODUCTION

Exposure to early adversity (EA) is associated with an increased incidence of various psychopathologies associated with dysregulated emotional and cognitive processes such as depression and anxiety (for a review, see Gershon et al., 2013), which are twice as prevalent in women compared to men (Bangasser and Valentino, 2014). By its very nature, EA leads to prolonged stress, increasing the secretion of stress hormones at an early age (cortisol in humans; Carlson and Earls, 1997; Fisher et al., 2000; Kertes et al., 2008; Bruce et al., 2009; Bernard et al., 2015).

Being liposoluble, cortisol easily crosses the blood-brain-barrier and binds to multiple brain regions, notably those that support cognitive processes that are crucial in stress regulation: the hippocampus (Herman et al., 2012), the amygdala (van Stegeren et al., 2007), and the prefrontal

cortex (Diorio et al., 1993). The hippocampus is important for explicit (i.e., declarative) memory formation and in the consolidation (Morris, 2007) and contextualization (Wiltgen et al., 2006) of information to be encoded. The amygdala is responsible for the detection of negative emotions and for threat detection (Bishop, 2008), and the prefrontal cortex plays a key role in emotion regulation processes through its functional inhibitory connection to the amygdala (frontoamygdala connectivity; Wager et al., 2008). These brain regions also play a critical role in the regulation of the physiological stress system: the hippocampus (Herman et al., 2012) and the prefrontal cortex (Diorio et al., 1993) both inhibit it, whereas the amygdala activates it (Herman et al., 2003).

A wealth of studies have assessed cognitive functions sustained by these regions in children and adults exposed to EA (for a review, see Lupien et al., 2009). Results show impaired declarative memory in trauma-exposed children (Ayoub et al., 2009; Bos et al., 2009; Carrión et al., 2010), and increased threat detection in children exposed to trauma relative to non-exposed children (Pollak et al., 2000; Fries and Pollak, 2004; Cicchetti and Curtis, 2005; Parker and Nelson, 2005). A study reported that this effect may be amplified in girls as opposed to boys (Soe et al., 2018). For prefrontal cortex and frontoamygdala connectivity functions, studies reported decreased abilities of emotion regulation in children exposed to trauma (Maughan and Cicchetti, 2002; Marusak et al., 2015; McLaughlin et al., 2015). All these effects seem to be long lasting, with studies reporting decreased declarative memory (Majer et al., 2010), increased threat detection (Nicol et al., 2014; Russo et al., 2015), and decreased emotion regulation abilities (Abravanel and Sinha, 2015) in clinical samples of adults reporting EA during childhood. However, so far, this pattern of altered cognition in adulthood has mostly been reported in subjects suffering from a psychopathology (such as anxiety or depression; Clark et al., 2010), making it difficult to determine whether the impaired cognitive processes result from EA or from the pathology.

Interestingly, these three brain regions that are sensitive to stress hormones follow different developmental trajectories. While the volume of the hippocampus expands from birth until the age of 2, the amygdala develops from the first year of life until the age of 20, and the prefrontal cortex as well as the frontoamygdala connectivity mainly develop between the ages of 8 and 29 (Giedd et al., 1996; Lupien et al., 2009). The age of 8 years old therefore represents an important milestone in brain development, as the establishment of the frontoamygdala connectivity allows for the independent inhibition of the amygdala by the prefrontal cortex during threat perception (Lupien et al., 2009). Recent studies have shown that before the development of the frontoamygdala connectivity (i.e., before 8 years old), the parent plays a critical role in regulating the child's stress response during threat perception, although this "buffering effect" is dampened when being reared in early adverse conditions (Gunnar et al., 2015).

The differential development of the brain regions sensitive to stress hormones has led to the "Life Cycle Model of Stress", which suggests that there may be early windows of vulnerability during which specific regions of the developing brain are most sensitive to stress hormones produced in response to environmental influences (Lupien et al., 2009). Exposure to stress and/or adversity during these key vulnerable periods could modulate the development of those brain regions for the duration of the adversity. The Life Cycle Model of Stress implies that the age at which an individual is exposed to EA for the first time (namely, the "minimal age at exposure") could lead to different physiological and/or cognitive outcomes depending on the brain regions that are developing at the time of exposure.

Given the importance of the hippocampus, the amygdala and the prefrontal cortex on regulating the activity of the physiological stress system (Lupien et al., 2009), in a first study, we tested the Life Cycle Model of Stress by measuring the effects of minimal age at exposure on the activity of diurnal and reactive cortisol levels (Raymond et al., 2021). We also compared this model (i.e., minimal age at exposure) to the classical "Accumulation model" of EA, which stipulates that the number of EA predicts patterns of cortisol dysregulations in adulthood. Results showed that although the number of EA was not associated with patterns of basal or reactive cortisol secretion, adults first exposed to EA between the ages of 3 and 7–an important time window for amygdala development–showed greater cortisol awakening response and lower cortisol reactivity relative to those first exposed to EA before 3 or after 7 (Raymond et al., 2021).

The minimal age at exposure could also be used to predict the nature of the cognitive patterns that will result from EA. For example, first exposure to adversity during the first year of life (hippocampal development) could result in later (adult) declarative memory impairment, whereas first exposure to adversity at the age of 7 years old (amygdala development) would result in increased threat perception. On the other hand, exposition to EA at 10 years of age could impact prefrontal cortex and lead to decreased frontoamygdala connectivity development that would translate in difficulties in emotion regulation in adulthood. Although the effects of EA on cognitive functions of children and adults have been assessed in previous experiments, no study to date has tested the effects of minimal age at exposure to EA on cognitive functions sustained by the hippocampus, the amygdala, and the frontoamygdala connectivity in adulthood.

The purpose of the current study was to test the Life Cycle Model of Stress by measuring the effects of minimal age at exposure on cognitive processes of healthy adults as a function of whether individuals were first exposed to EA during the development of the hippocampus, the amygdala and the frontoamygdala connectivity. The Life Cycle Model of Stress predicted that (1) individuals first exposed to EA during the development of the hippocampus would present decreased declarative memory performance in adulthood; (2) individuals first exposed to EA during the development of the amygdala would present increased threat detection in adulthood compared to the other groups, and (3) individuals who were first exposed to EA during the development of the frontoamygdala connectivity would present decreased emotion regulation abilities in adulthood as opposed to the other groups. Furthermore, given the sex-discrepancy in stress-related

psychopathologies in adulthood, we expected that these effects would be greater in women as opposed to men.

MATERIALS AND METHODS

Participants

Fifty-three naturally cycling women and 32 men aged 21–40 (M = 28.28, ±5.56) participated in a two-session protocol, occurring 14 days apart. Participants were recruited from the greater Montreal region through advertisements asking for healthy adults exposed to diverse forms of EA. Internet advertisements and posters on University campus were used. Interested participants contacted a study staff member and were screened over the phone to make sure that they did not suffer from any physiological (neurological, cardiovascular disease, and general health problems) or psychological (ex. diagnosed depression, schizophrenia, and personality or anxiety disorders) conditions that could influence the results. Participants did not take medication and oral contraceptive use was also an exclusion criterion for women. Participants were screened for EA over the phone (see section "Assessment of early adversity: Adverse Childhood Experience Questionnaire"). Eligible participants were then scheduled for two visits at the laboratory, at a 2-weeks interval period, between 1:30 p.m. and 4:30 p.m to control for the circadian cycle of cortisol and for synchrony effects on cognition (May and Hasher, 1998).

Moreover, given that we also took measurements of physiological stress (results published in Raymond et al., 2021), all women performed the first testing session during the follicular phase of their menstrual cycle (based on women self-reports on their last menstruation and their menstrual cycle length) given the known interaction between the hypothalamic-pituitary-adrenal axis and the hypothalamic-pituitary-gonadal axis. This procedure was implemented so that women would be in the luteal phase of their menstrual cycle 2 weeks later when they back to the laboratory for a follow-up session where they underwent the *Trier Social Stress Test* in order to test their cortisol reactivity, and given the well-known effects of sex hormones on salivary free cortisol levels (Kirschbaum et al., 1999).

Questionnaires

Assessment of EA: Adverse Childhood Experience Questionnaire

The international version of the Adverse Childhood Experiences Questionnaire (ACE-Q; World Health Organization, 2014) was administered to all eligible participants to assess exposure to EA. The ACE-IQ, which was administered during phone screening, is a validated 13-item questionnaire assessing physical, sexual, and emotional abuse, neglect, environmental disaster, household dysfunctions such as witnessing violence, parental separation, death or mental illness, substance abuse, bullying, as well as collective and community violence. The participant must answer "yes" or "no" to each item. To assess the minimal age at exposure to EA, we added one question to every item of the ACE-IQ. Therefore, participants answering that they were exposed to a subtype of adversity were asked to specify at what age the specific EA type began. In order to calculate the time elapsed since last exposure, participants were also asked at what age it last happened (which was calculated as the subtraction of the age at last exposure from the age at the time of testing).

Given the fact that exposition to EA has been shown to lead to various stress-related psychopathologies associated with decreased emotion regulation abilities in adulthood, we compared the three groups on depressive symptoms, trait anxiety, as well as explicit (i.e., trait) emotion regulation strategies using the following questionnaires.

Beck Depression Inventory

Depressive symptoms were assessed with the Beck Depression Inventory-II (BDI-II; Beck et al., 1988), which is a 21-item self-reported questionnaire assessing depressive symptoms that occurred in the past 2 weeks. The BDI assesses depressive symptoms with statements ranging from 0 to 3 in terms of intensity. A meta-analysis showed that this instrument has a test–retest reliability ranging from r = 0.60–0.83, and a Cronbach's alpha of 0.81 for non-psychiatric participants (Beck et al., 1988). In this study, we used the sum score of the BDI-II, with higher scores indicating more severe symptoms.

State-Trait Anxiety Inventory for Adults

Trait anxiety was assessed with the State-Trait Anxiety Inventory for adults (STAI-Y; Spielberger, 1983). This questionnaire consists of 40 items and is divided into two subscales: Trait and State anxiety. The Trait subscale measures anxiety as a personality trait. As a result, these questions must be answered in a general sense and not with regards to a particular situation. This subscale consists of 20 questions that are answered on a scale ranging from 1 to 4 (1 meaning « hardly ever » and 4 being « almost always »), where a high score indicates a high level of anxiety in general. In this study, only the sum score of the Trait subscale was used. The test–retest reliability for the Trait subscale is r = 0.86 and the internal consistency is 0.90 (Spielberger, 1983).

Affective Style Questionnaire

In order to measure dispositional emotion regulation, participants filled out the Affective Style Questionnaire (Hofmann and Kashdan, 2010). The ASQ is a 20-item instrument that measures individual differences in emotion regulation abilities. Each item is measured on a 5-point Likert scale. The questionnaire is composed of three subscales: "Concealing" (which refers to habitual attempts to conceal or suppress affect), "Adjusting" (a general ability to manage, adjust, and work with emotions as needed), and "Tolerating" (an accepting and tolerant attitude toward emotion). The scale is reliable (r = 0.062) and has good internal consistency (Concealing α = 0.84, Adjusting α = 0.82, and Tolerating α = 0.68).

Cognitive Assessment

To answer our primary research questions, we selected cognitive tasks sustained by the three brain regions involved in stress regulation (hippocampus, amygdala, and prefrontal cortex) and that are therefore likely to be influenced by chronic stress following exposure to EA.

Each task was programed using E-Prime (Version 3.0; Psychology Software Tools, 2016) and was conducted on a Dell Inspiron 6000, 400 MHz laptop computer with a 15-inch monitor. Observations were made in a seated position, at a distance of 60 cm from the screen.

Declarative Memory

A word list of 20 neutral words controlled for word length, frequency of use and grammatical category was created by our team. This type of words list has been commonly used to assess declarative memory performance in other studies (Eichenbaum, 2004; Bird and Burgess, 2008; Canamar and London, 2012; Jeneson and Squire, 2012). Each trial began with a fixation cross presented for 500 ms, followed by the stimulus (i.e., word) for 2,000 ms. Words were presented one by one, printed in black (*Arial*, font size 24) and presented twice randomly. Three free recalls were conducted: (1) immediately after word presentation; (2) 20 min later, and; (3) 2 weeks later during the second testing session. Between the immediate and the +20 min recall, participants were asked to read magazines, waiting for the next task. At each recall phase, participants were given 5 min to remember as many words as possible and write them down. This type of task has been shown to rely on hippocampal activity.

Attentional Bias Toward Threat

In order to assess attentional bias toward threat (vs. neutral ones), we used a modified version of the *Posner spatial orienting paradigm* (Posner, 1980) that we previously used in another study assessing attentional biases (for task overview, see Pilgrim et al., 2010). The task began with a black cross on a white background, which remained on the screen. Afterward, a word (Arial black font, point size of 24) was presented for 200 ms on either the right or left hand side of the screen. This word was either positive (i.e., calm), threatening (i.e., judged), or neutral (i.e., measure) and served as a stimulus cue to the location of a subsequent target. A black target asterisk appeared immediately after the word cue disappeared and remained on the screen for 1,000 ms. Subjects were told to respond as quickly and accurately as possible regarding the spatial location of the target. They pressed one key if it appeared on the right (M) side of the black cross and another if it was shown on the left (Z).

Given that the main purpose of this task was to verify whether groups differed in terms of their attentional biases toward threatening cues as opposed to neutral ones, we only analyzed the valid trials for threatening and neutral cues. Hence, when negative words related to threat were previously presented, a more rapid response (as opposed to neutral ones) on the target indicated an attentional bias toward threatening information. As such, the reaction time for negative words was subtracted from the reaction time to neutral words to create a ratio (Pilgrim et al., 2010). Hence, a negative ratio indicates an attentional bias toward threat.

There was a total of 540 trials with 360 valid trials (66%), i.e., when the cue and the target appeared on the same side of the display, assessing attentional engagement. There were 180 invalid trials (33%), i.e., when the cue and the target appeared on opposite sides of the display, examining attentional disengagement. An additional 48 animal word cue trials were embedded within regular trials and participants were instructed to refrain from responding. These animal word cue trials were included: (1) to reduce the generation of automaticity and (2) to increase the degree of attention paid to the semantic nature of words (these were not analyzed in the current study). Another 15 separate practice trials (Ekman, 1976) incorporating different word cues than the experimental trials were presented at the start of the task. This type of task measuring attentional biases toward threat (vs. neutral ones) trough reaction time measurements has been shown to rely on the activity of the amygdala (Goldstein et al., 1996; Pasley et al., 2004; Vuilleumier and Pourtois, 2007; Tottenham and Sheridan, 2010; Méndez-Bértolo et al., 2016; Mier et al., 2017).

Emotion Regulation

The automatic regulation of emotion processing was assessed with the *Emotional conflict task* (Etkin et al., 2015). The task consisted of 160 presentations of happy or fearful facial expression photographs drawn from the set of Ekman and Friesen (Ekman, W.V. Friesen, Pictures of Facial Affect, Consulting Psychologists, Palo Alto, CA; 1976). Photographs were overlaid with "HAPPY" or "FEAR" written in red letters (see **Figure 1** for task overview). Subjects were instructed to identify as quickly and accurately as possible the underlying facial emotion of visual stimuli (happy or fearful), while inhibiting the reading of the overlying word (happy or fear). Trials varied in congruency in such a way that some trials were "congruent" (i.e., non-conflict, expression of actor matched the word) and others were "incongruent" (i.e., conflict, expression of actor did not match the word). One hundred and sixty (160) trials were presented: half (80 trials) were congruent, and the other half (80 trials) were incongruent. As previous studies have demonstrated, incongruent trials induce an emotional conflict, which is associated with slower reaction times, increased activation of the prefrontal cortex, and decreased activation of the amygdala (Etkin et al., 2015). Stimuli were presented for 1,000 ms, with a varying interstimulus interval of 2,000–4,000 ms (mean = 3,000 ms), in a pseudorandom order and were counterbalanced across trial types for expression, overlying word and sex of figures. Response times were included in the analyses for all correct trials with a reaction time between 200 and 1,200 ms (see **Figure 1** for task overview). Conflict regulation was measured by contrasting reaction time for "congruent followed by incongruent" trials (cI trials) to "incongruent followed by incongruent" trial (iI trials). This kind of task has been shown to rely on the top-down inhibition of the amygdala by the prefrontal cortex, both critical structures involved in the emotion regulation system (Etkin et al., 2015) and in stress response (Lupien et al., 2009).

General Protocol

The ethics committee of the *Research Center of the Institut universitaire en santé mentale de Montréal* approved this study and all participants provided written informed consent to take part in the study. Upon their arrival at the laboratory for their first visit, and after giving their written consent, participants were

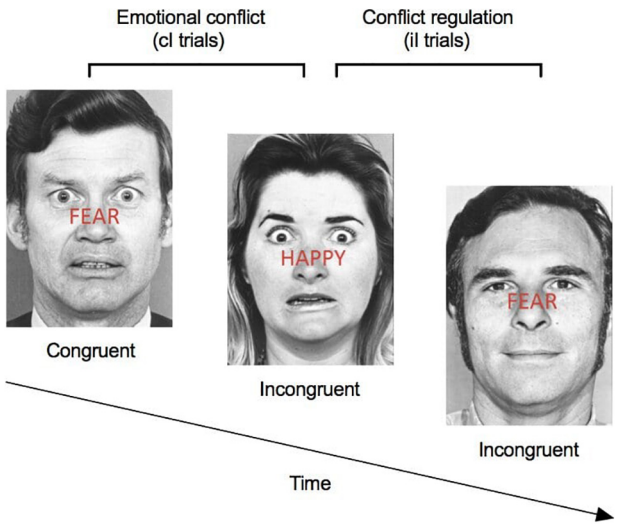

FIGURE 1 | Emotional conflict task. Participants were instructed to identify as quickly and accurately as possible the underlying facial emotion (happy or fearful), while inhibiting the reading of the overlying word (happy or fear). Trials varied in congruency such that some trials were "congruent" (i.e., non-conflict, expression of the actor matched the written expression) and others were "incongruent" (i.e., conflict, expression of the actor did not match the written expression). One-hundred sixty (160) trials were presented: half were congruent and half were incongruent. Conflict regulation was measured by contrasting reaction time for "congruent followed by incongruent" trials (cI trials) to "incongruent followed by incongruent" trial (iI trials).

first asked to complete the cognitive tasks (declarative memory, modified version of the *Posner paradigm*, and the *Emotional conflict task*). In order to control for a potential fatigue effect, the order of the tests was randomly counterbalanced between participants. Following the first session, participants were asked to complete the questionnaires (BDI-II, STAI-T, ASQ, and others that were not analyzed in the current study) at home via the Studies Web Automation Tool (SWAT), which is a secured web platform developed by the Center for Studies on Human Stress (Montreal, QC, Canada). Since we wanted to ensure confidentiality, participants were given individualized secure codes to log in and access the online questionnaires.

After 2 weeks, participants were asked to come back to the laboratory at the same time as their first visit that had occurred 14 days earlier. Upon their arrival at the lab, they were asked to remember as many words as possible from the first session (third free recall of the declarative task; see section State-Trait Anxiety Inventory for Adults). Participants thereafter completed additional tasks during this session, though they will not be discussed within the context of the current paper.

Initial Treatment of the Data

Group formation: Minimal age at exposure to EA: Based on the information collected during the phone interview, we categorized the participants in three different groups based on the age at which the first EA occurred: between 0 and 2 years old (during hippocampal development), thereafter named "Infancy"; between 3 and 7 years old (during amygdala development), thereafter named "Early childhood" or after 8 years old (frontoamygdala connectivity development) thereafter named "Childhood/Adolescence". In a first study, we have demonstrated that this model predicted both basal and reactive cortisol levels, and that this model was a better statistical fit as opposed to the classical "Accumulation" model of EA which stipulates that the number of EA predicts patterns of cortisol dysregulations in adulthood (see Raymond et al., 2021; for statistical details and Goodness of fit indices).

Statistical Analyses

Data were examined for potential outliers via studentized residuals, with residuals $\geq \pm 3.29$ considered outliers. Two participants exhibited extreme score on the Posner task in the 0–2 years old group (one above and one below average). One participant exhibited extreme (above average) scores on the BDI in the 8+ years old group and two participants on the STAI-T in the 3–7 years old group (one above and one below average). In line with ethical considerations, participants who had a score of 14 or more on the BDI (criteria for mild depression; Beck et al., 1988) were contacted by the main investigator and provided with psychological resources. Analyses were run twice: once including the Winzorized values, and once excluding them. Since no difference was found between the two sets of analyses, Winzorized data were included in the final analyses. The distribution of our variables was also assessed for skewness and kurtosis prior to conducting the statistical analyses. Using indices for acceptable limits of ± 2, data were found to be normally distributed (George and Mallery, 2010).

First, we conducted an ANOVA to assess whether groups differed in terms of their scores on the BDI-II, STAI-T and the three subscales of the ASQ. For cognitive processes, we first assessed whether groups (3) differed on overall task accuracy and reaction time (modified version of the *Posner paradigm* and *Emotional conflict task*). Thereafter, we conducted an ANOVA for attentional biases (reaction times in the modified version of the *Posner paradigm*) and on the conflict regulation ratio (reaction time for iI–cI) with groups (3: minimal age at exposure 0–2 years old; 3–7 years old – +8 years old) and sex (2: women and men) as the between subject factors. We also verified whether groups differed in declarative memory using a repeated measures ANOVA with time (3 recalls: immediate – +20 min – +2 weeks) as the within subject factor and groups (3: minimal age at exposure 0–2 years old; 3–7 years old – +8 years old) and sex (2: women and men) as the between subject factors. Significant interactions were decomposed and Bonferroni corrections were applied when multiple comparisons were conducted during *post hoc* analyses.

RESULTS

Preliminary Analyses

In our sample, the mean score on the ACE-IQ was 4.6 (min = 1; max = 11; ± 2.25). Analyses confirmed that each group differed from each other in minimal age of exposure [$F(2,84) = 223.450$; $p < 0.001$] (all $ps < 0.001$). Analyses also revealed group differences in accumulation of EA (ACE-Q summed score)

[F(2,84) = 5.119; p = 0.008], with the "Childhood/Adolescence" group presenting lower accumulation of EA as opposed to the "0–2 years old" group (p = 0.007). Groups did not differ on the time elapsed since last exposure to EA [F(2,84) = 0.698; p = 0.501], nor in age [F(2,84) = 0.272; p = 0.763] (see **Table 1**). Given group differences on the ACE-Q, and to ascertain that obtained group differences as a function of minimal age at exposure did not pertain to this potential confounding factor, we re-conducted every significant analyses while including the ACE-Q summed score as a covariate.

Depressive Symptoms

The ANOVA revealed no main effect of group [F(2,84) = 1.640; p = 0.201], nor a group × sex interaction [F(2,84) = 0.600; p = 0.552] (see **Table 2** for further details).

Trait Anxiety

The ANOVA revealed no main effect of group [F(2,84) = 1.224; p = 0.300], nor a group × sex interaction [F(2,84) = 0.999; p = 0.373] (see **Table 2** for further details).

Trait Emotion Regulation

For the "concealing" subscale of the ASQ, the MANOVA revealed no main effect of group [F(2,84) = 0.432; p = 0.651], nor a group × sex interaction [F(2,84) = 1.949; p = 0.150]. For the "adjusting" subscale of the ASQ, the MANOVA revealed no main effect of group [F(2,84) = 1.871; p = 0.161], but a group × sex interaction [F(2,84) = 3.461; p = 0.036]. *Post hoc* analyses revealed group differences in men [F(2,32) = 3.661; p = 0.039], with those in the 0–2 years old group presenting decreased scores on the "adjusting" subscale as opposed to those in the 3–7 (p = 0.032) and 8+ (p = 0.016) years old groups, which did not differ from one another (p = 0.921; see **Figure 2**). No group differences were found in women [F(2,52) = 0.441; p = 0.646]. For the "tolerating" subscale of the ASQ, the MANOVA revealed no main effect of group [F(2,84) = 0.457; p = 0.635], nor a group × sex interaction [F(2,84) = 0.592; p = 0.556] (see **Table 1** for further details).

Cognitive Processes

Declarative Memory

The ANOVA performed on declarative memory performance revealed a significant main effect of time [F(2,136) = 234.557; p < 0.0001], with each timepoints differing from one another (encoding: M = 11.38, SD = 2.85; +20 min: M = 10.33, SD = 2.95; 2 weeks post encoding: M = 6.73, = SD; all ps < 0.0001). A main effect of sex was found [F(2,68) = 0.535; p = 0.002], with women presenting increased recall at all timepoints as opposed to men (**Figures 3A,B**). No main effect of group [F(2,68) = 0.803; p = 0.452], no time × group interaction [F(4,136) = 0.738; p = 0.568], nor a time × group × sex interaction [F(4,136) = 1.308; p = 0.275] was found.

Attentional Bias Toward Threat

The ANOVA revealed no main effect of group [F(2,84) = 1.684; p = 0.192], but a significant group × sex interaction [F(2,84) = 3.785; p = 0.027]. *Post hoc* analyses revealed group differences in women [F(2,52) = 4.349; p = 0.018], with women in the Childhood/Adolescence group presenting greater attentional bias toward threat as opposed to the Infancy (p = 0.01) and Early childhood (p = 0.017) groups, which did not differ from each other (p = 0.998). No group differences were found in men [F(2,32) = 0.924; p = 0.408] (**Figure 2A**). We re-conducted the analysis while including the ACE-Q summed score as a covariate, which did not modify the obtained group × sex interaction [F(2,83) = 3.734; p = 0.028]. As shown in **Figure 2A**, a negative value indicates a faster reaction time to negative relative to neutral words, indicating an attentional bias toward threat.

Emotion Regulation

As expected, responses to congruent trials were significantly faster as opposed to incongruent trials [t(93) = 58.543, p < 0.0001]. However, the ANOVA revealed no main effect of group [F(2,84) = 0.833; p = 0.439] nor a group × sex interaction [F(2,84) = 2.901; p = 0.061] (**Figure 2B**).

DISCUSSION

The purpose of this study was to test whether the cognitive processes sustained by the brain regions involved in the stress response are modulated by the age at first exposure to EA. We found that although groups did not differ on declarative memory performance and emotion regulation, women who were first exposed to EA after the age of 8 ("Childhood/Adolescence": during the development of frontoamygdala connectivity) presented increased attentional bias toward threat as opposed to those who were first exposed between 0 and 2 years old ("Infancy": during the development of the hippocampus) or between 3 and 7 years old ("Early childhood": during the early development of the amygdala).

The absence of a significant group difference in declarative memory performance could be taken as suggesting that declarative memory is not associated to the age at first exposure to EA. Yet, a few studies found altered verbal memory in traumatized children (Bos et al., 2009) and in adult women exposed to EA and suffering from post-traumatic stress disorder (Bremner et al., 2003, 2004). However, these results were obtained in clinical populations, raising the question as to whether the memory deficits observed were due to exposition to EA or to the underlying psychopathology. The absence of a control group in our study prevents us from answering this important question. Be this as it may, the absence of a group difference in declarative memory performance could also be explained by a number of factors. First, they could be due to the task that we used. It is possible that the word list was not difficult enough given that we recruited resilient/healthy participants (none of them were suffering from a psychopathology) and that 62% of them came from University settings, which promotes the retention of verbal material (Guerra-Carrillo et al., 2017). Another possibility is that our negative finding on the declarative memory task is due to a practice effect, especially in women as they presented greater memory performance when compared to men. It would be interesting to replicate these results with a different task (such as spatial memory), that is still associated with declarative memory.

TABLE 1 | Demographic information and health behaviors as a function of minimal age at exposure.

	Infancy	Early childhood	Childhood/Adolescence	*p*
N (women)	30 (23)	25 (15)	30 (15)	
Age	27.73 (5.20)	28.32 (6.11)	28.80 (5.58)	0.575
Minimal age at exposure to EA	0.27 (0.64)*	4.36 (0.95)*	10.10 (2.84)*	<0.001
ACE-Q sum score	5.36 (2.20)	4.84 (2.49)	3.63 (1.73)*	0.008
Health behaviors				
Alcohol intake (# of drinks/week)	3.25 (3.91)	2.40 (3.72)	2.79 (2.76)	0.773
BMI	24.94 (3.32)	23.03 (2.71)	24.25 (3.73)	0.250

Each panel depicts the number of participants for each group (with the number of women), as well as the mean (and corresponding standard deviation) for the following variables: age (in years), minimal age at exposure to EA (in years), sum score to the ACE-Q. Asterisk (*) indicates significant between-group differences. ACE-Q, Adverse Childhood Experience Questionnaire; EA, early adversity; MAE, minimal age at exposure; SD, standard deviation.

TABLE 2 | Socio-emotional characteristics as a function of minimal age at exposure and sex.

	Infancy		Early Childhood		Childhood/Adolescence		*p*
	Men	Women	Men	Women	Men	Women	
Beck depression inventory-II	11.57 (2.84)	10.09 (1.64)	6.33 (2.50)	9.64 (2.00)	6.29 (2.01)	7.53 (1.94)	0.580
State trait anxiety inventory - trait	47.57 (1.51)	49.76 (0.87)	50.56 (1.33)	50.29 (1.07)	50.93 (1.09)	49.93 (1.03)	0.369
Affective style questionnaire							
Concealing	28.00 (2.50)	23.67 (1.34)	27.67 (2.21)	21.36 (1.77)	23.14 (2.98)	24.93 (1.70)	0.079
Adjusting	17.43 (2.08)*	21.53 (1.20)	23.56 (1.83)	19.79 (1.47)	23.79 (1.90)	21.27 (1.42)	0.039*
Tolerating	16.71 (1.14)	16.90 (0.66)	17.00 (1.01)	15.64 (0.81)	17.64 (0.98)	16.27 (0.78)	0.612

*No group differences were found on the Beck Depression Inventory, the Trait subscale of the State Trait Anxiety Inventory nor on the three subscales of the Affective Style Questionnaire. A group by sex interaction was found on the Adjusting subscale of the Affective Style Questionnaire. *$p < 0.05$. Mean (SD).*

FIGURE 2 | Attentional biases and emotion regulation as a function of minimal age at exposure and sex. **(A)** Increased attentional biases toward threatening information in women in the "Childhood/Adolescence" group as compared to men in the "Childhood/Adolescence" group, women in the "Early childhood" and women in the "Infancy" group. A negative value indicates a faster reaction time to negative words relative to neutral words and therefore suggests an attentional bias toward threat. **(B)** Emotion conflict regulatory ability in individuals who were first exposed to EA during "Infancy" (0–2 years old), "Early childhood" (3–7 years old) or "Childhood/Adolescence" (after 8 years old). Negative values indicate faster response for il relative to cl trials (il-cl) and suggest appropriate emotion regulation. *$p < 0.05$. Error bars represent SEM. MEA, minimal age at exposure; y/o, years old.

Interestingly, we found that adult women who were first exposed to EA after the age of 8 presented increased attentional bias toward threat as opposed to the other groups. In line with the Life Cycle Model of Stress (Lupien et al., 2009) one possible interpretation for this finding may be that first exposure to EA during the development of the prefrontal cortex (while the frontoamygdala connectivity is taking place), could result in a diminished capacity of the frontal cortex

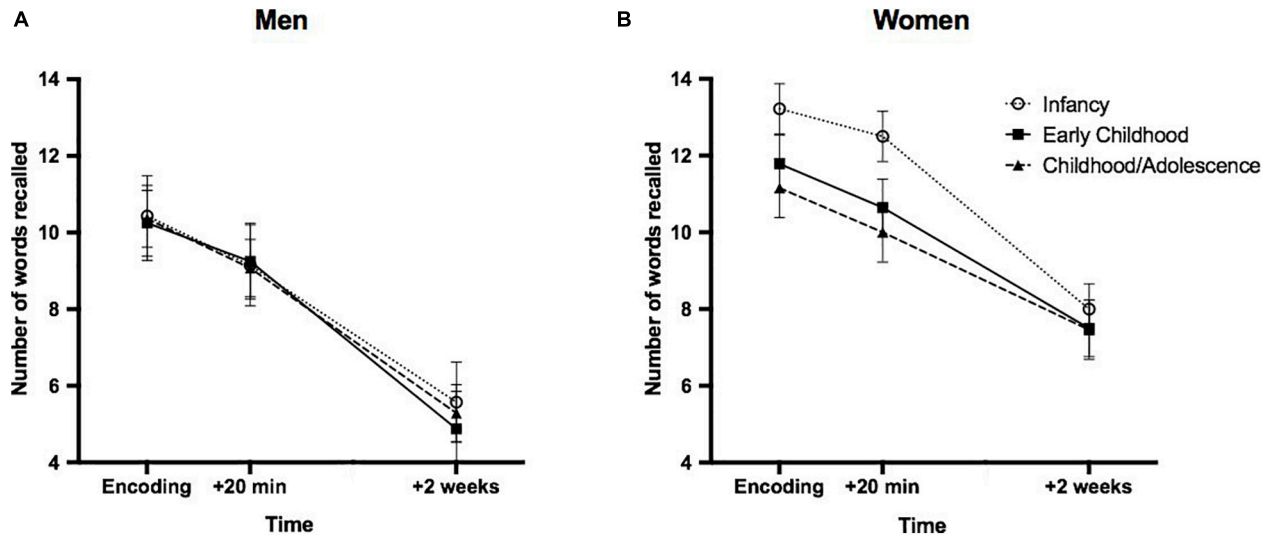

FIGURE 3 | Performance on the declarative memory task as a function of minimal age at exposure and sex. Declarative memory in men **(A)** and women **(B)** first exposed to EA during "Infancy" (0–2 years old), "Early childhood" (3–7 years old) or "Childhood/Adolescence" (after 8 years old). A main effect of sex was found, with women presenting an overall increased word recall as opposed to men. Error bars represent SEM.

to inhibit the amygdala during threat perception. Although attentional biases to threat are typically thought to rely on the activity of the amygdala alone, neuroimaging studies have shown that the prefrontal cortex plays a critical role in down-regulating the activity of the amygdala during the processing of threatening stimuli (Browning et al., 2010; Angelidis et al., 2018), allowing disengaging the attention away from threat (Peers et al., 2013). In individuals who first undergone EA *after* 8 years of age, a reduced frontoamygdala functional connectivity may limit the inhibition of the amygdala and result in increased attentional biases toward threat. In support of this idea, recent neuroimaging studies found reduced frontoamygdala connectivity in adolescents (Cisler, 2017; Park et al., 2018) and adults exposed to EA (Javanbakht et al., 2015), although the age at first exposure to EA was not taken into account in these studies. Soe et al. (2018) also found that a reduced frontoamygdala connectivity was more prominent in girls as opposed to boys exposed to EA, which could explain the obtained sex differences in our sample.

The increased attentional bias in women first exposed to EA after the age of 8 is also in line with the Stress Acceleration Hypothesis (Callaghan and Tottenham, 2016), which proposes that EA accelerates the development of the fear circuitry in order to adapt to harsh surroundings and therefore confers an important evolutionary advantage in adverse, stressful environments (Callaghan and Tottenham, 2016). Studies have provided data supporting the Stress Acceleration Hypothesis (Callaghan and Tottenham, 2016) by showing that EA accelerated the development of the frontoamygdala connectivity in children exposed to parental deprivation in such a way that prefrontal cortex-amygdala interactions were more "adult-like" following EA when compared to children not exposed to parental deprivation (for a review, see Gee et al., 2013). When studied in a laboratory context, such differences in cognitive processes may be perceived as abnormal. However, new data by Ellis et al. (2017) suggest that these cognitive changes could be evolutionary adaptive so that EA may shape social and cognitive abilities in order to better adapt to a threatening environment. For example, if a child is reared in an early violent environment, it may be evolutionary adaptive to take a longer time to regulate a negative affect or to present an attentional bias toward threat, as it may allows quicker reaction to threat (Shackman et al., 2007). However, such cognitive adaptation could lead to the development, maintenance and/or exacerbation of vulnerability to stress and anxiety in adulthood (for a review, see Raymond et al., 2018).

The current study, combined with a previous psychoneuroendocrine study conducted on the sample of healthy adults exposed to EA, suggest that minimal age at exposure might influence whether or not the acceleration of the frontoamygdala connectivity occurs. Indeed, in a previous study, we supported the Stress acceleration hypothesis by showing that first exposure to EA during the development of the amygdala (between 3 and 7 years old) led to increased cortisol awakening response and decreased cortisol reactivity to a laboratory psychosocial stressor as opposed to those first exposed between 0 and 2 or after 8 years old (Raymond et al., 2021). This study therefore suggests that first exposure to EA *during* the development of the amygdala but *before* the frontoamygdala connectivity is developing adaptively alters the activity of the hypothalamic-pituitary-adrenal axis (Raymond et al., 2021). In the current study, we suggest that being exposed to EA for the first time *after* 8 years of age (during development of frontoamygdala connectivity) may prevent the acceleration of the frontoamygdala connectivity, resulting in decreased prefrontal cortex inhibition of the amygdala during threat perception.

From a clinical perspective, it is also possible to believe that first exposure to EA during late childhood/adolescence leads to long-lasting (clinical or subclinical) cognitive vulnerability to anxiety in women. Indeed, various studies have suggested that adolescence represents a vulnerable window in the development of various anxiety disorders accompanied by an increased attentional bias toward threat (Derryberry and Reed, 2002) such as social phobia, panic disorder, agoraphobia and generalized anxiety disorder, all of which are twice as prevalent in women as opposed to men (Beesdo et al., 2009). Although further studies are needed in order to better understand the specific mechanism underlying this increased increase incidence of anxiety disorders in adolescence, studies have suggested that an interaction between individual factors (genetic, neurobiological, and temperament) combined with chronic stress (such as EA) could be at play (for a review, see Beesdo et al., 2009). Here, we suggest that healthy women who did not (or not yet) develop anxiety disorders following exposition to EA during adolescence might still present cognitive vulnerabilities that are typically found in children, teenagers and adults who suffer from such psychopathologies. It would be interesting for future studies to investigate the psychosocial, endocrine and neurocognitive protective factors that might prevent the development of psychopathologies in adolescence following exposure to EA. By doing so, interventions could focus on promoting these protective factors following exposition to EA in youth.

We also report that groups did not differ in implicit emotion regulation as measured by the *Emotional conflict task* (Etkin et al., 2006), a task believed to be sustained by the prefrontal cortex and frontoamygdala connectivity. Theoretically, if exposure to EA after the age of 8 leads to poorer frontoamygdala connectivity as proposed above, then adults from the group exposed to EA after the age of 8 should have presented an impaired performance on this task. However, it has to be noted that very few studies have provided cognitive data on the *Emotional conflict task* in populations exposed to EA, and it is possible that this task does not tap entirely on cognitive processes sustained by prefrontal cortex. To this day, only one study has found that children exposed to trauma performed poorly on the *Emotional conflict task* when compared with matched non-exposed children (Marusak et al., 2015). Second, the complexity of the network implying emotion regulation (Goldin et al., 2008) could explain the absence of a group difference on the *Emotional conflict task*. Indeed, although the prefrontal cortex and the amygdala are the main brain structures involved in emotion regulation, a wide array of other brain regions are also important in order to allow such regulation to occur (Goldin et al., 2008). One of these brain regions is the hippocampus (Zhu et al., 2019), which begins to develop at birth (Lupien et al., 2009), allowing the contextualization of emotional cues in order to regulate the affect (Goldin et al., 2008). It is therefore possible that being exposed to EA during early development may also affect the ability to regulate emotions in adulthood. In support of this idea, we found that men exposed to EA between 0 and 2 years-old ("Infancy": development of the hippocampus), presented decreased scores on the "Adjusting" subscale of the *Affective Style Questionnaire* (Hofmann and Kashdan, 2010), which suggests a reduced tendency to reappraise negative emotions.

Our study contains a number of limitations that need to be addressed. First, although we found promising results in terms of the cognitive processes affected by minimal age at exposure to EA, the absence of neuroimaging measures does not allow us to discuss the mechanism(s) underlying our findings. It would be interesting to conduct neuroimaging studies in order to compare frontoamygdala connectivity of adults who were first exposed to EA before and after the age of 8. This would help in further supporting the interpretation of our findings. Second, although self-reported measures of EA have been demonstrated to be valid and reliable [for a review, see Hardt and Rutter (2004)], it is possible to believe that our measure of minimal age at exposure might have been biased by a poor memory recollection of the events. That could be especially the case when assessing events that occurred during the first years of life. Third, although our limited sample size did not allow to address this specific question, it would also be interesting for future studies to investigate whether the nature of EA impact the obtained results. Fourth, the absence of a control group (with a summed score of 0 on the ACE-Q) limits our interpretation of the results. Finally, our sample was predominantly composed of women and sex was not distributed equally across groups. It is possible that the sex differences we observed results from this imbalance in groups composition as opposed to a differential effect of minimal age at exposure to EA in men and women.

To conclude, our results partly support the Life Cycle Model of Stress and suggest that exposure to EA after 8 years old leads to attentional biases in adult women. Drawing on the core assumptions of the Stress acceleration hypothesis (Callaghan and Tottenham, 2016), we argued that such differences could be due to the chronic secretion of stress hormones following first exposure to EA during the development of the frontoamygdala connectivity. This finding is of high importance, given that adolescence was repeatedly shown to be a critical window in the development of anxiety disorders in women. Here, we suggest that exposition to EA during this critical period might lead to a long-lasting cognitive vulnerability to anxiety in healthy women. While this suggests that EA might be an important environmental factor in the development of cognitive patterns associated with anxiety in women, it also raises the need to further investigate the protective factors that made these individuals resilient toward the disease. By doing so, we could optimize treatment and, ultimately, implement preventive measures for this vulnerable population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Research Center of the Institut Universitaire en Santé Mentale de Montréal. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR and SL designed the study protocol. CR, VW, A-AJ, and CL collected the data. CR, SL, and M-FM analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Amygdala Allostasis and Early Life Adversity: Considering Excitotoxicity and Inescapability in the Sequelae of Stress

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Early life adversity (ELA), such as child maltreatment or child poverty, engenders problems with emotional and behavioral regulation. In the quest to understand the neurobiological sequelae and mechanisms of risk, the amygdala has been of major focus. While the basic functions of this region make it a strong candidate for understanding the multiple mental health issues common after ELA, extant literature is marked by profound inconsistencies, with reports of larger, smaller, and no differences in regional volumes of this area. We believe integrative models of stress neurodevelopment, grounded in "*allostatic load*," will help resolve inconsistencies in the impact of ELA on the amygdala. In this review, we attempt to connect past research studies to new findings with animal models of cellular and neurotransmitter mediators of stress buffering to extreme fear generalization onto testable research and clinical concepts. Drawing on the greater impact of inescapability over unpredictability in animal models, we propose a mechanism by which ELA aggravates an exhaustive cycle of amygdala expansion and subsequent toxic-metabolic damage. We connect this neurobiological sequela to psychosocial mal/adaptation after ELA, bridging to behavioral studies of attachment, emotion processing, and social functioning. Lastly, we conclude this review by proposing a multitude of future directions in preclinical work and studies of humans that suffered ELA.

Keywords: amygdala, stress, neurodevelopment, brain, adversity, development, allostatic load

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INTRODUCTION

The amygdala has been the focus of a great deal of attention in research aimed at understanding the effects of Early Life Adversity (ELA). The fact that this evolutionarily ancient brain structure is of interest is perhaps not surprising given this region's essential role in socioemotional functioning (Bachevalier et al., 1999; Amaral, 2002) and that forms of ELA (e.g., child abuse; child neglect) engender problems with regulating the emotions and behaviors (Kessler et al., 2010). In this review, we first discuss the basic functions and development of this brain region, noting why this area has been a strong candidate for understanding the multiple mental health issues common after ELA. We next explore past research focused on this brain region in human and preclinical models of

ELA exposure and potential of an allostatic load model to disentangle apparent inconsistencies in these findings. We then extend this idea, pulling from parallel models put forth in research studies focused on autism and neurodevelopment, and integrating preclinical rodent and nonhuman primate findings, to make specific hypotheses about human behavioral and clinical correlates of specific cellular and neurotransmitter changes. We finally close this document with proposals for future research directions connected to these ideas.

DEFINING EARLY LIFE ADVERSITY AND REVIEWING CONNECTIONS BETWEEN ELA AND POOR MENTAL HEALTH

Surveying work on ELA, researchers have focused on different samples exposed to adversity including child maltreatment (e.g., physical or sexual abuse), extreme household dysfunction (e.g., having a parent with a severe mental illness), and poverty (alongside lower "social standing"). These and related negative experiences have been referred to using different umbrella terms, such as "early life stress," "child trauma," "toxic stress," "early adversity," and "Adverse Childhood Experiences (ACEs)." An important starting question is whether to lump adversities together, think about specific experiences (e.g., early social neglect or physical abuse), or examine potential shared dimensions of ELAs. Initial research took a purely cumulative exposure approach, summing up the total number of adversities suffered, or looking at child trauma *across* different forms of maltreatment (Felitti et al., 1998; Hanson et al., 2012a; Gorka et al., 2014). This approach has high explanatory power and can deal with the common pattern of co-occurrence between many forms of adversity (Appel and Holden, 1998; Emery and Laumann-Billings, 1998; Kellogg and Menard, 2003); however, cumulative models provide less clarity about potential mediating mechanisms. More recently, starting frameworks (Belsky et al., 2012; McLaughlin et al., 2014a) argue for the difference between dimensions of adversity (i.e., harshness vs. unpredictability; deprivation vs. threat) to advance mechanistic understanding of the impact of ELA. Moving forward, the field must strike a balance between more mechanistic approaches and the reality of the high co-occurrence of different ELAs, as well as the low-base rates for an isolated form of adversity. In this space, there are multiple reviews about this topic (e.g., McLaughlin et al., 2020; Smith and Pollak, 2020) and we would direct readers to those past publications for more in-depth discussion. Here, we take a more broad and inclusive definition of ELA. This is in keeping with many preclinical approaches and the well-known ACEs study from the Centers for Disease Control and Prevention. These experiences share some core elements in that they can be psychosocial hazards, are severe deviations from the expected environment, and activate stress responsive physiology (as thoughtfully discussed by Nelson and Gabard-Durnam, 2020). We, however, return to this issue in later sections of this document.

While definitions are variable, clear from a large body of research is that multiple forms of ELA are associated with compromised development and long-term physical and mental health challenges (Shonkoff et al., 2012). Across different models and forms of ELA, a rigorous body of work has established a strong connection between these experiences and antisocial and aggressive behavior (or so-called "*externalizing psychopathology*"). For example, greater disruptive behavior and conduct problems have been found in victims of child sexual abuse (Mallett and Schall, 2019), in individuals who suffer physical abuse or neglect (Moylan et al., 2010; Muniz et al., 2019), and in youth from households with lower income (Votruba-Drzal, 2006; Evans and Cassells, 2014; Piotrowska et al., 2015). Turning to depression, anxiety, and other forms of "*internalizing*" psychopathology, similar patterns have been noted, with major depressive disorder (MDD) being associated with child maltreatment (Nanni et al., 2012; Björkenstam et al., 2017) and to a lesser, though still significant, extent after exposure to poverty (Letourneau et al., 2013; Peverill et al., 2020). ELAs are often associated with a more severe and chronic course of MDD (Chapman et al., 2004; Wiersma et al., 2009; McLaughlin et al., 2011; Carr et al., 2013), as well as poorer response and remission outcomes for the treatment of this disorder (Williams et al., 2016). Examined collectively, research has consistently linked ELA with a plethora of negative mental health outcomes, with risk commonly increasing with each additional exposure (Felitti et al., 1998). Understanding the potential mechanisms by which ELA worsens mental health, as well as candidate mechanisms of resilience and recovery, is critical to prevention, intervention, and ultimately curative treatments.

THE AMYGDALA AS AN IMPORTANT SOCIOEMOTIONAL HUB: CONSIDERATION OF BASIC FUNCTIONS AND NEURODEVELOPMENT

Situated in the anterior portion of the temporal lobe, the amygdala is a complex of subcortical nuclei important for the evaluation of the emotional significance of incoming stimuli (Davis and Whalen, 2001). While the constituent amygdala subnuclei each subserve different functions (*described later in this document*), collectively the amygdala calculates the intensity of response to positive and negative emotional stimuli (Ambroggi et al., 2008; Fox et al., 2015). Because of its connections to evaluative regions in frontal cortex, contextual information from hippocampus, procedural and reward information from striatum, and autonomic outputs to the hypothalamus and ascending cholinergic nuclei, the amygdala can mediate adaptive physiological (e.g., autonomic reactivity) and behavioral (e.g., reallocation of attentional resources) responses to varied environmental and social challenges (Phelps, 2004; Hariri, 2009). In line with these ideas and its involvement in fear learning, meta-analyses of functional neuroimaging studies in humans find the amygdala is activated by a number of negative emotions (Lindquist et al., 2012), with direct stimulation of human amygdala confirming the primacy of fear and anxiety (Lanteaume et al., 2007).

Given these basic functions, research focused on different forms of psychopathology have centered on the amygdala. Various mood and anxiety disorders [e.g., MDD; generalized

anxiety disorder (GAD); post-traumatic stress disorder (PTSD)] and some samples with autism have shown greater amygdala *activation* to facial displays of fear and anger (Etkin and Wager, 2007; Hamilton et al., 2012). Differences in amygdala *structure* have also been noted in individuals with excessive socioemotional responses ranging from autism (Nacewicz et al., 2006; Kim et al., 2010), to MDD, to social phobia to PTSD (Karl et al., 2006; Woon and Hedges, 2009). Examined collectively, these different bodies of research underscore the amygdala as central to emotion processing, with aberrant structure and activity in multiple forms of psychopathology.

Thinking about the amygdala and neurodevelopment, it is important to note that nuanced work has begun to illustrate that the typical development of the amygdala is non-linear in nature, similar to overall cortical development (Shaw et al., 2006), with amygdala development continuing well into adulthood. Substantial post-natal development may mean that environmental experience has a greater potential to significantly impact and influence neurodevelopment. The basic structural architecture of the amygdala is well-established at birth, but volumes increase significantly during infancy (Humphrey, 1968; Ulfig et al., 2003). Though some cross-sectional reports suggest a general decrease in volume during adolescence and in early adulthood, longitudinal quantitative MRI work (Wierenga et al., 2014), as well as analyses focused on amygdala histology (Cunningham et al., 2002; Saul et al., 2014), suggest a more complex pattern. This work indicates amygdala volumes *relative to brain volume* continue to increase through adolescence, reaching maximum volumes in the late teens or early twenties. The exact age of these peaks is, however, dependent on the sex and pubertal dynamics of an individual (Goddings et al., 2014). Such trajectories fit with preclinical work finding active periods of cell proliferation in these regions during adolescence (Saul et al., 2014; Sorrells et al., 2019) and continued development in human post-mortem studies (Avino et al., 2018)

In sum, research underscores that the amygdala is central to emotion processing, and its abnormal structure and predominantly excessive activity are common to different forms of psychopathology. Furthermore, the amygdala displays rapid structural growth early in life, with continued refinement of this anatomy into adolescence and early adulthood. Variations in outcomes, both behaviorally and neurally, may be due to ELA impinging upon core developmental processes happening at the specific time of stress exposure. These core functions and neurodevelopmental trajectories are important bedrocks to consider when thinking about the effects of ELA on amygdala structure and potential critical periods for important affective processes, such as the buffering against generalization of fear.

WHAT IS THE STATE-OF-SCIENCE OF ADVERSITY'S IMPACT OF THE AMYGDALA? WHAT MIGHT BE CAUSING THESE INCONSISTENCIES?

Surveying preclinical research, as well as studies in human samples, it is clear that stress exposure and exposure to adverse experience impacts amygdala structure; however, the magnitude and directionality of these effects has been challenging to understand and to cohesively summate. In regards to preclinical work, these studies, primarily conducted in late juvenile or early adult rodents, has found exposure especially to restraint stress leads to volumetric *increases* such as dendritic arborization in amygdala nuclei (Vyas et al., 2002; Mitra et al., 2005; Cohen et al., 2013); this is opposite the hippocampal changes where dendritic retraction is typically seen after stress (Watanabe et al., 1992; Magariños et al., 1996, 1997). Initial work in human adults did not find alterations in amygdala structure in samples exposed to ELA (Bremner et al., 1997; Cohen et al., 2006). A recent study, however, noted larger amygdala volumes in adults who were exposed to higher levels of cumulative stress during childhood (Evans et al., 2016). This was in contrast to a large study of non-demented older adults ($N = 466$) that found participants who reported two or more early-life events had significantly smaller amygdalae with increasing age (Gerritsen et al., 2015). When adults had a history of ELA exposure and comorbid psychopathology (such as PTSD or borderline personality disorder), smaller amygdala volumes have typically been reported in ELA-exposed samples (Driessen et al., 2000; Schmahl et al., 2003; Weniger et al., 2008; Irle et al., 2009; Veer et al., 2015; Souza-Queiroz et al., 2016). However, in a unique sample of adolescents and adults with elevated risk for psychosis, no associations between adversity and amygdala volumes were found (LoPilato et al., 2019).

Structural neuroimaging in human pediatric populations have, similarly, yielded mixed results. In children exposed to neglect, research reports have noted larger amygdalae (Mehta et al., 2009; Tottenham et al., 2010; Roth et al., 2018), as well as no differences (Sheridan et al., 2012; McLaughlin et al., 2014b; Hodel et al., 2015). Child poverty has been associated with larger (Noble et al., 2012) and with smaller amygdalae (Luby et al., 2013; Ellwood-Lowe et al., 2018). Smaller amygdalae (Edmiston et al., 2011; McLaughlin et al., 2016), as well as no differences, have been found in adolescents who experienced child maltreatment (De Bellis et al., 1999, 2001, 2002; Carrion et al., 2001; Gold et al., 2016). Similarly, exposure to community violence during childhood was related to smaller amygdala volumes (Saxbe et al., 2018; Weissman et al., 2020); however, related recent work did not replicate this association in a similar sample (Butler et al., 2018).

Our research group attempted to deal with some of these inconsistencies by using a rigorous tracing protocol and focusing on three different forms of ELA—child poverty, physical abuse, and early social neglect—in a sample of youth ages 9–14. This work also deployed rich measures of stress exposure, obtained through semi-structured interviews with both youth and parents. Interestingly, while reduced amygdala volumes were common to all types of ELA and not statistically differentiable at our sample size, the impact of low SES was greatest with physical abuse slightly worse than institutional neglect (Hanson et al., 2015b). A portion of these differences could reflect our finding that greater cumulative stress exposure was associated with smaller amygdala volumes. Recently, Herzog et al. (2020) tried to compare the impact of different types of ELAs using cutting-edge statistical methods (random forest regression) and found neglect during childhood and adolescence was related to smaller amygdala

volumes. However, work using latent class models to identify classes of ELA (e.g., Family Instability; Direct Victimization) did not find any associations between ELA type(s) and amygdala volume (King et al., 2019). Future studies with large samples that *are equally matched on stress severity* could potentially differentiate unique contributions of ELA type.

Across these studies, one major limitation is that the preponderance of this work has been cross-sectional in nature. Such work can miss the complexity of neurobiological trajectories, underscoring the importance of studying development longitudinally (Shaw et al., 2006; Wierenga et al., 2014). In regard to longitudinal samples, Whittle et al. (2013) found childhood maltreatment was associated with slower growth of the left amygdala, but these associations reversed if participants presented with psychopathology. Mirroring some of these patterns, VanTieghem et al. (2021) used an accelerated longitudinal design to compare youth who previously suffered early social neglect in institutional care and a comparison sample without such ELA. Youth who suffered early social neglect had a reduced growth rate of the amygdala, resulting in smaller volumes by adolescence. Given the panoply of inconsistent findings reviewed here, it will be important to judiciously walk through potential sources of measurement error and biological/physiological variance as the field continues to think about connections between ELA and neurodevelopment.

Surveying the human neuroimaging studies focused on ELA, there is a wide-range of variation in methodology, sampling strategies, and conceptualizations of ELA and related stress. Each of these areas likely interjects inaccuracies and biases in reported results. At a basic level, volumetric quantification of the amygdala is more complex, and potentially inaccurate, than many may allude to. Volumetric amygdala measurement can be performed using manual and automated protocols. A good deal of the early structural imaging work employed manual tracing of the amygdala; this methodology can often be more precise and accurate, but is time-consuming and requires extensive expertise. In our own work at the University of Wisconsin-Madison, even with 8 months of training, 80–90% of undergraduate trainees failed to reach spatial and numerical reliability on our whole amygdala segmentation, and expert tracing still requires at least 2 h per amygdala (e.g., Caldwell et al., 2015). This is now amplified by a factor of six or more, as we hand-trace individual subdivisions and subnuclei. Manual tracing can still be problematic if poorly executed, evidenced by over constraint (i.e., highly precise but insensitive to individual variation) or simple drift in technique leading to high variability (e.g., low intraclass correlation coefficients). For example, Cohen et al. (2006) reported that the average amygdala volume for an adversity-exposed group was 1.27 and 1.16 mL (for the right and left amygdala), while a non-adversity exposed group was 1.26 and 1.15 mL (for the right and left amygdala). These groups were not significantly different from one another. However, rarely highlighted is that the error for these measures was actually higher than the mean volumes (ELA group = 1.40 for right, 1.28 for the left; Comparison group = 1.43 for right, 1.36 for the left). This suggests inconsistent and problematic hand-tracing, and similar results (*null or otherwise*) with these patterns should likely be greeted with skepticism. Moving away from hand-tracing, there are now many commonly available automated methods for amygdala volumetric quantification (e.g., Hanson et al., 2012b; Buser et al., 2020; Liu et al., 2020). These approaches represent a scalable and easy-to-deploy method to potentially test relations between volumetric measures and psychological variables of interest; such methods may be particularly important given that structural MRI-datasets are exponentially increasing in size (from 10 to 1,000 s). However, many approaches (i.e., *Freesurfer*) often yield unsatisfactory results with high-variability and low-validity (Babalola et al., 2009; Morey et al., 2009; Dewey et al., 2010; Hanson et al., 2012b). For example, we found that automated segments of the amygdala generated by Freesurfer had low bivariate correlations with volumes from rigorous hand-tracing of the same structure (Left $r = 0.563$, Right: $r = 0.560$; (Hanson et al., 2012b). Particularly damning, in Hanson et al. (2015b), Freesurfer-estimated amygdala volumes captured neither group differences nor individual differences in cumulative life stress in a sizable sample of youth who suffered different forms of ELA

For high-throughput studies, a new generation of automated segmentation tools is required. In our recently published approach (Liu et al., 2020), accurate amygdala acquisition and segmentation required modifications in both a Multi-Atlas model and a Convolutional Neural Network. Multi-Atlas models match overall context, as cost is calculated across the whole brain, but requires hyperbolic exaggeration of subtle boundaries to distinguish the amygdala subnuclei. In contrast, the neural network easily matches fine details, but requires combination with a parallel network constraining the model on a larger contextual scale. Either of these dual-scale approaches is acceptable, but all segmentations require additional visual quality checks.

Turning to issues with study designs, many investigations in humans have had a large age range of participants (e.g., 5–15 years old in studies focused on pediatric populations); this is particularly important to note given amygdala developmental trajectories reviewed earlier. For example, LoPilato et al. (2019) examined a large cohort of individuals, but the age range spanned from 12 to 30 years of age. During this span, amygdala structure is actually increasing in volume, hitting a peak volume, and possibly shrinking again; mixing of age groups likely occludes associations between ELA and volume. Connected to this, in most work, age is simply added as a linear covariate to statistical models. Research might think of alternative strategies for studies where participants span multiple developmental epochs (or large age ranges, i.e., individually fitting a quadratic term of age). For instance, Merz et al. (2018) examine the interaction of age and family income/poverty, one type of ELA, in a sizable cohort of youth ($N = 296$). When these investigators examined the full cohort, there were no significant effects detected. But, looking at age X ELA interactions, these investigators found that lower family income was significantly associated with smaller amygdala volumes in adolescence (13–21 years old). However, this relation was not seen for younger age children (3–12 years), suggesting important neurodevelopmental associations may only be revealed when considering ELA and developmental stage(s).

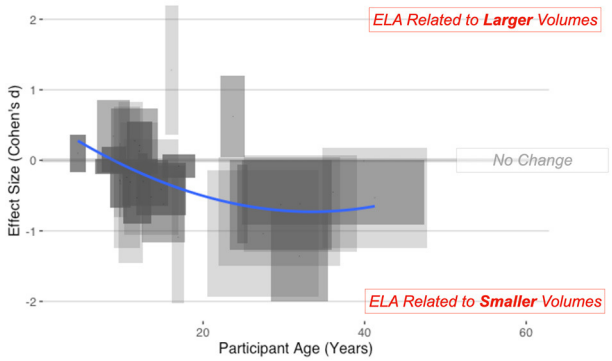

FIGURE 1 | Combining across automated and manual methods for quantifying the amygdala, here we depict amygdala volumetric differences for ELA-exposed samples. Cohen's d, with 95% confidence intervals (CIs) of effects, are shown on the vertical axis, while the span in age is shown on the horizontal axis. Longer boxes (on the horizontal axis) indicate studies with larger age-ranges in their samples, while wider boxes (on the vertical axis) depict studies where the effect size estimates and 95% CIs span a larger numeric range. Study sample size is also depicted in this graphic with lighter boxes being studies with smaller sample sizes and darker boxes representing studies with a larger number of participants. Individual study data is available online at: https://github.com/jlhanson5/Hanson_Nacewicz_Frontiers_Amygdala_Review_Data.

We believe that these confusing results can be explained by the inverted-*U* allostatic growth trajectory. High levels of stress initially increase amygdala volume, but the most extreme (or chronic) levels of adversity may result in smaller volumes. Support for this idea comes from multiple avenues. First, cross-sectional studies suggest complex associations between amygdala structure, the intensity of ELA, and developmental consequences of stress. For example, Mehta et al. (2009) found larger amygdalae in children exposed to early social neglect; however, the duration of early neglect (that these same children were exposed to) was actually related to smaller amygdalae. Similarly, combat-exposed adults with PTSD exhibited larger amygdalae compared with their non-PTSD counterparts. But, in individuals with a history of ELA and PTSD, smaller amygdala volumes were actually found (Kuo et al., 2012). A recent multi-group study by Morey et al. (2016) that examined maltreated youth with PTSD, without PTSD, and non-maltreated healthy volunteers further highlights this. Maltreated youth without PTSD demonstrated larger amygdalae compared with maltreated youth with PTSD and compared with non-maltreated control youth. However, PTSD symptoms were correlated with amygdala volumes, with greater symptomatology being related to smaller volumes.

This pattern is visibly evident looking across multiple studies (**Figure 1**) considering age of the sample and the direction of effect. For younger samples (<9 years of age), there is reasonable data to show volumetric increases in the amygdala, but looking at adult samples, there is the suggestion of smaller volumes. In past meta-analyses, there has often been aggregation of different studies but limited consideration of a non-linear trajectory. This is perhaps why there has been conflicting results across different meta-analyses. Taking a more thoughtful developmental perspective supports this inverted-*U* pattern of alterations. For example, recent longitudinal work (Whittle et al., 2013) suggest a slowing of growth of the amygdala after ELA. Particularly important to highlight, youth who suffered early social neglect, one form of ELA, had a reduced growth rate of the amygdala, resulting in smaller volumes by adolescence (VanTieghem et al., 2021) and it is as-yet unknown if this represents a delay or a missed critical period to learn fear and safety.

Looking at ELA as a form of allostasis raises many testable questions. Consider the measurement and definition of ELA. These concepts are notoriously difficult to measure and may take many forms. For example, in samples exposed to poverty, in addition to challenges with low income, there are often greater residential neighborhood problems in impoverished environments (Steptoe and Feldman, 2001). Higher crime, inadequate neighborhood services, and transportation problems may constitute sources of chronic stress. There are also more daily "mundane" stressors in low SES environments and this may contribute to greater rates of psychopathology (Kanner et al., 1981; Almeida, 2005; Odgers and Jaffee, 2013). Indeed, as Slavich (2019) noted the large preponderance of life stress exposure work is "*measuring only the superficial contours of this complex construct*." There is a massive and significant variation in severity, frequency, timing, and duration of adversity, and as yet the relative weight of these against disruptions of parental attachment is unknown. Each of these factors could likely be introducing heterogeneity in the large body of findings we reviewed above. As we discuss later in this document, it is likely that different forms of ELA may share phenomenological elements (e.g., experiences of threat; McLaughlin et al., 2014a).

"AMYGDALA ALLOSTASIS:" FOCUSING ON EXCITATORY/INHIBITORY NEUROCHEMISTRY AND CONSIDERING BEHAVIORAL CONSEQUENCES AFTER ELA

When considering the potential neurodevelopmental impact of ELA, it is critical to realize that: (a) the amygdala is not a unitary brain area, but rather heterogenous subnuclei with unique functions and developmental trajectories (as detailed in **Figure 2**); and (b) stress may exert non-linear effects in concordance with McEwen's notions of "allostatic load." Connected to heterogeneity, volumetric growth during development varies across subnuclei, but is driven by an increase in neurons of the basal nuclei and lateral nucleus. Other subnuclei of the amygdala (i.e., the paralaminar region) gradually loses putative newly generated/differentiated neurons, suggesting a proliferative role (or migration pathway) akin to subventricular zones of neighboring structures (Chareyron et al., 2012; Avino et al., 2018; Jurkowski et al., 2020). Related to allostatic, inverted-*U* patterns, allostatic load translates roughly to "a new normal" and is the detrimental physiological consequence caused by sustained excessive activation of stress-responsive systems (Danese and McEwen, 2012); this is commonly caused by chronic or repeated exposure to psychosocial stressors.

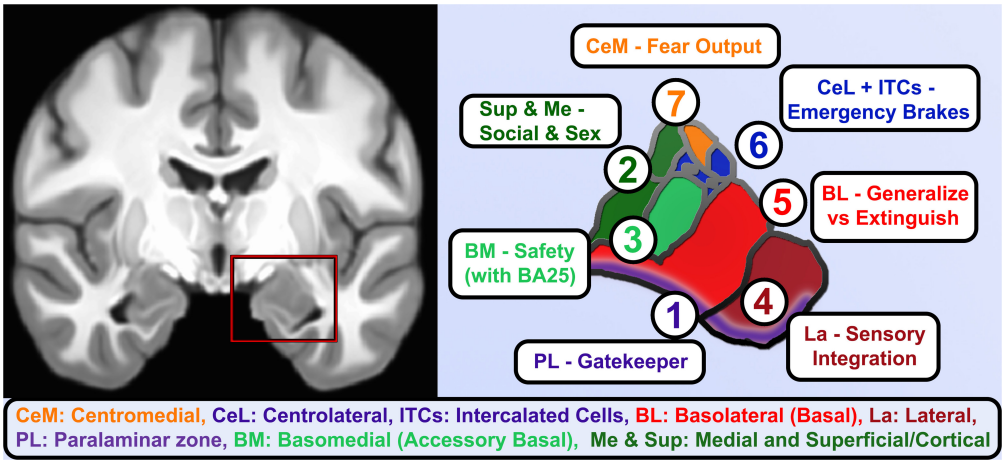

FIGURE 2 | Functional roles of human amygdala subnuclei. A coronal view of T1-weighted structural MRI (left) showing amygdalae resting on the anteriormost hippocampus and separated by the white matter of the alveus, and closeup of hand-segmented amygdala subnuclei (right). (1) While there is still debate about whether the paralaminar region is a true nucleus vs. the subventricular region of other nuclei, it is the main zone of from which newborn neurons migrate into the basal and lateral nuclei and houses dopamine-innervated GABAergic cells that gate activation of the basal and lateral nuclei. (2) The corticial nucleus and superficial nuclei are closely linked to the olfactory system and in vomeronasal animals coordinate responses to pheromones. As such, these and the medial nucleus of the amygdala contribute to latent drives such as recognition of conspecifics, maternal attachment, and sex-related differences and behaviors. (3) Basomedial nucleus is an early-developing nucleus that bears some functional similarities to the adjacent superficial nuclei, e.g., changing serotonin receptor expression in studies of early maternal separation, but also brings in information about safety cues from higher centers through direct innervation by infralimbic/BA25 projections. (4) The lateral nucleus gathers information about threatening cues and contexts from highly processed sensory information and contextual information from hippocampus. It is the largest nucleus in humans and most reliably enlarges in allostatic load, consistent with rodent studies showing dendritic expansion as fear generalizes. (5) The Basolateral or simply Basal nucleus similarly expands volume and dendrites after inescapable stress, but it is also home of key "extinction cells" that integrate information from other nuclei and prefrontal inputs and feeding forward inhibition to contextualize or extinguish threat responses and turn off dopamine from the VTA. (6) The intercalated cell islands (ITCs) and about half the cells in the lateral division of the central nucleus (CeL) receive feedforward inhibition from basal nuclei or are directly activated by the social bonding hormone oxytocin (CeL), to inhibit latent and previously learned fear responses. They receive heavy dopaminergic innervation and send effectors to centromedial (CeM) and a similar lateral-to-medial inhibition of the ascending arousal signaling of the cholinergic basal nucleus of the stria terminalis. About half the neurons are so-called "fear on" neurons that signal latent and previously-learned fears with some threat signals from innervation by the paraventricular thalamus. (7) CeM sends long-range projections that tonically inhibit hypothalamic and brainstem autonomic centers until fear and safety signals integrated by CeL shifts toward threat and inhibits these neurons.

With stress and ELA exposure, we specifically believe that increases in cellular complexity in the amygdala (e.g., higher dendritic branching; increased synaptogenesis) leads to excessive excitation, untamable by inhibitory synapse growth, and this cascades to excitotoxic damage and ultimately cell death (schematized in **Figure 3**). Connecting these two elements, we believe that basolateral portions of the amygdala largely encode cues, contexts and behaviors mapping the boundary between *safety* and known threats.

In regard to allostatic load of the amygdala and nearby structures, recent preclinical work shows that neurogenesis in hippocampus is highly constrained by the metabolic costs of deviating from an optimal ratio of excitatory to inhibitory neuronal firing (Wang et al., 2020). Considering metabolic costs of excitation-inhibition ratios in the amygdala (**Figures 2**, **3**), the basolateral portions are primarily excitatory with a large concentration of glutamate (Glu) releasing neurons, but under resting conditions an extensive network of inhibitory, γ-aminobutyric acid (GABA) releasing neurons anchored in the paralaminar zone largely silences the basolateral complex (Quirk and Gehlert, 2003). Modulatory neurotransmitters (Marowsky et al., 2005) relieve this inhibition, bringing online the basal and lateral nuclei that compute the magnitude of feed-forward fear and safety signals and set the degree of fear generalization. Manipulating GABA in this region modulates amygdala reactivity and reduces anxiety and social behaviors (Sanders and Shekhar, 1995; Paulus et al., 2005; Del-Ben et al., 2012). Multiple forms of affective psychopathology are theorized to be related to excessive Glu-GABA ratio in this region (Sanders and Shekhar, 1995; Cortese and Phan, 2005; Pittenger et al., 2007; Tye et al., 2011). The basolateral complex ultimately sends information about conditioned and aversive stimuli to the centromedial "output" nuclei (Duvarci and Pare, 2014) which, like neighboring striatum, have among the highest density of GABA synapses in the brain (Sutoo et al., 2000). Central subnuclei integrate the ascending information with previously learned fear and social hormonal signals to ultimately trigger autonomic and behavioral fear responses through brainstem projections.

With increasing levels of chronic stress (such as in ELA), there is interruption of the normal excitation-inhibition balance in the amygdala. This may occur through multiple pathways and may explain a portion of the heterogeneity of structural results seen previously. First, stress may cause higher excitability in the basolateral amygdala, due to: increases in the number of spontaneously firing neurons (Zhang and Rosenkranz, 2012), enhanced excitatory synaptic drive (Padival et al., 2013),

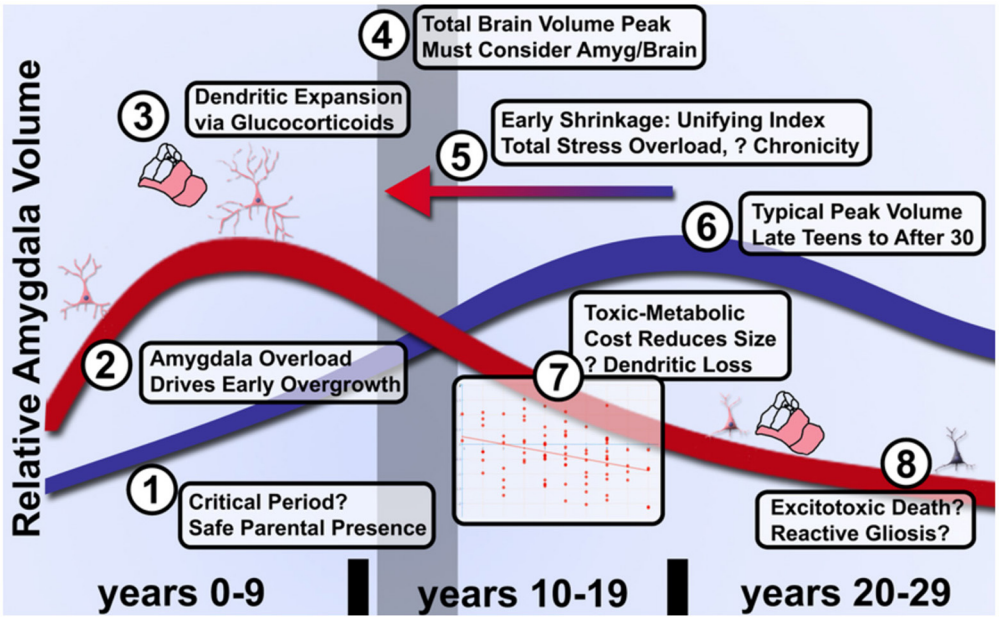

FIGURE 3 | Model of graded acceleration of amygdala volume relative to brain volume and regions of clarification needed in future studies of allostatic load in ELA. (1) It has not been established whether a critical period exists during the first several months of parental attachment or after during which parental presence facilitates overcoming adversity and an enduring sense of parent-cued safety. (2) As noted above, more evidence will be needed to clarify whether early enlargement or rate of growth is quantitatively linked to degree of subjective adversity. (3) While certainly involving glucocorticoids, dendritic expansion and a shift toward excitation over inhibition, the limits on peak volume and transition to shrinkage is poorly understood even in ASD. (4) These changes relative to control volumes must be further couched in an understanding of typical brain development that likely peaks by the preteen years, thus even a shift earlier in life (5) of the same curve could manifest as a distinct quadratic shape in proportion to severity of adversity. (6) Furthermore, the peak of typical amygdala development and influences on timing have not been fully elucidated due to the challenges of longitudinal study. (7) Provided individuals are assessed after the quadratic peak, amygdala shrinkage quantitatively reflects cumulative life adversity and better characterization of this pathophysiology may distinguish effective treatments from the natural course of disease. (8) Whether inflammation in the form of reactive gliosis and excitotoxic death occur and can be prevented will require better longitudinal tools that track microsctructure and neurochemistry longitudinally.

and the increased expression and activation of glutamatergic N-methyl-D-aspartate (NMDA) receptors (triggering so called "silent synapses"; Mozhui et al., 2010; Suvrathan et al., 2014; Tzanoulinou et al., 2014b). In addition, preclinical work indicates stress exposure can lead to GABAergic alterations. Stress during the juvenile period is related to changes in GABA-A protein expression (Jacobson-Pick and Richter-Levin, 2012; Tzanoulinou et al., 2014a) and reduction in enzymes involved with synthesis of GABA in the rodent amygdala (Tzanoulinou et al., 2014b). Interestingly, adversity during the juvenile period may actually result in an immature-like expression profile of the GABA-A receptor subunit (Jacobson-Pick et al., 2008). This may be compensatory, as stress leads to long lasting loss of tonic GABA-A receptor currents in the projection neurons of lateral amygdala (Liu et al., 2014). Finally, stress may cause alterations in cortisol, cannabinoids, and neuropeptides, such as cholecystokinin and neuropeptide Y (Shekhar et al., 2005); alterations in these systems may further indirectly impact excitation-inhibition balance in the amygdala (Hadad-Ophir et al., 2014; Radley et al., 2015). Examined collectively, stress impacts both inhibition and excitation in the amygdala through direct alterations in Glu and GABA, as well as through indirect stress-induced changes in hormonal and neuropeptide signaling. These multiple pathways tilt the amygdala to a more excitable state, paralleling the human findings of amygdala hyper-reactivity to emotional stimuli after exposure to ELA.

At a larger scale, one sees a more overall excitable amygdala, with increased dendritic spines in basolateral nuclei after adversity (Vyas et al., 2002, 2003, 2004, 2006; Mitra et al., 2005). Despite these neurobiological alterations, organisms exposed to stress must still strive to maintain homeostasis, regulating their physiological and behavioral responses to environmental experiences. This hyper-excitable state of the amygdala, however, has the potential to lead to wear and tear on the body and brain (allostatic load and overload; McEwen, 1998; McEwen et al., 2015). Thinking about these patterns, McEwen (2003, 2005) noted parallels with brain alterations in humans during initial episodes of major depression, where larger volumes and increased functional activity of the amygdala have been noted (Frodl et al., 2003). McEwen further suggested that this hyperactivity might give way to eventual shrinkage, citing reports of smaller amygdalae after repeated depressive episodes (Sheline et al., 1999). Knitting together work in stress exposed juvenile animals, one sees preliminary support for this idea. Work from Rosenkranz et al. found repeated stress increases the excitability of amygdala neurons (Hetzel and Rosenkranz, 2014), but loss of spines in the amygdala after repeated stress during early development (Padival et al., 2015). Particularly interesting,

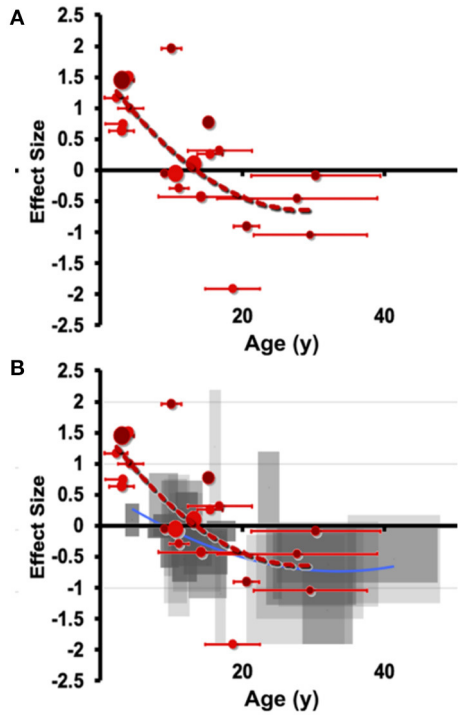

FIGURE 4 | Meta-analytic evidence for a transdiagnostic allostatic curve. Manual hand-tracing studies of **(A)** amygdala volume differences in individuals with autism (ASD) compared to those with autism. Effect size (hedges g) is shown on the vertical axis, with age shown on the horizontal axis (error bars indicate SD of age). Effect sizes are small volume corrected or corrected with total brain volume or intracranial volume. Of note, these samples meet the stricter DSM-IV represented by the smallest reported subgrouping by age. **(B)** Overlaying with the less steep curve of right amygdala from ASD shows a highly similar pattern of shrinkage that is at least as early as the onset seen in ELA.

animals resilient to stress had markers of reduced excitatory drive from glutamatergic inputs in the amygdala.

This pattern of early overgrowth is mirrored in individuals suffering their first episode of psychosis or affective psychosis (combined bipolar and unipolar depression), suggesting a common amygdala response in disorders of sustained distress (Velakoulis et al., 2006). Breaking this down further, Suor et al. (2020) recently showed that early overgrowth was evident in a cross-section of individuals with GAD, social anxiety (or both) and found that a component of social threat was associated with amygdala enlargement. We believe this to be a quantifiable transdiagnostic response to sustained evaluation of social threat, but capturing sizable cohorts suffering their first mood episode before treatment impedes our knowledge of the early stages of the allostatic response. Similarly, emotional overload during social situations is a hallmark of autism spectrum disorders (ASD), which have extensive characterization of degree of social impairment and, conveniently for scientific study, amygdala hyperactivation is expected to start at birth. In ASD, volumetric overgrowths have been reported early in development, but smaller volumes have been noted later in life (Nacewicz et al., 2006; Schumann and Amaral, 2006; Mosconi et al., 2009; Kim et al., 2010) and the rate or degree of early life overgrowth or later life shrinkage is associated with quantitative social impairments. Connected to these ideas, we show in **Figure 4** an aggregration of studies in individuals with autism, as well as ELA-exposed samples. We believe the clear pattern of overgrowth and shrinkage in ASD lays the groundwork for a dose-dependent acceleration of this pattern in ELA, consistent with the studies described above showing that adversity with psychopathology or more severe stress from adversity is frequently associated with smaller volumes even on a background of ELA-induced enlargement.

Returning to the case of high ELA exposure, early life volumetric expansion of the amygdala is likely coupled with higher functional reactivity and excitatory tone. Amygdala cells may be able to maintain a moderately high-load state, and individuals with this neurobiological phenotype may display higher levels of depression, anxiety, or other affective illnesses. In some, symptoms may not always reach clinical diagnostic thresholds, or the individuals may be less impaired, albeit with limited capacity to absorb another traumatic insult. In the case of particularly extreme stress exposure, occurring for longer durations of time, initial overgrowth and high metabolic strain may give way to subsequent volumetric shrinkage. In a way, the amygdala may reach a breaking point of over-excitation that leads to smaller structural volumes, while higher functional reactivity and excitatory tone are still present. This fits with the smaller volumes typically noted in adult samples exposed to stress, echoing rodent hippocampal models predicting that *high ratios of inhibitory neurons are metabolically more costly* if unable to reduce high firing rates of excitatory neurons (Wang et al., 2020). As such, we predict that individuals with the largest volumes in early childhood and smallest volumes after the first decade of life will manifest highest levels of symptomatology, likely presenting with one or more full-blown clinical diagnoses.

If amygdala volume after ELA *does* fit the allostatic model, what does this tell us about mechanisms of human psychopathology and treatment potential? What is lost when an the amygdala shrinks but maintains a pathological hyperexcited state? Our understanding of allostatic load grows out of a literature on "learned helplessness," essentially giving up in the face of a challenge, a core construct in stress-induced depression thought to bridge animal studies and major depressive disorder induced by overwhelming stress (Maier and Seligman, 1976). The key manipulation inducing learned helplessness was a systematic series of alternating mild stressors each day, with the key feature being unpredictability of the next day's stress [as reviewed by Maier and Watkins (2005)]. Sapolsky et al. had identified "damage" to hippocampal neurons (atrophy and loss of apical dendrites) in a case series of vervet monkeys that appeared to die of health consequences of social stress (Uno et al., 1989), but reproducing these with a reliable laboratory model of behavioral stress was *only* achieved using repeated immobility stress (also called "*restraint stress*"; Watanabe et al., 1992). Head to head comparisons with repeated unpredictable stressors showed immobility stress to cause greater dendritic loss in these cells, despite one of the unpredictable stresses on the commonly-used protocol being a single episode of immobility stress (Nibuya et al., 1999). McEwen's group showed antidepressant treatment could reverse hippocampal changes and related spatial

learning deficits (Conrad et al., 1996), but, more importantly, a sensitization to fear learning was untouched by the treatment. These investigators concluded "*the results indicate a powerful effect of repeated restraint stress on another brain region, possibly the amygdala, which overrides any influence of the hippocampus*" (Conrad et al., 1999). Vyas et al. built on the sensitized fear conditioning after stress and discovered increased dendritic length, branching, and spines (spines typically represent high fidelity excitatory synapses) throughout the amygdala (Vyas et al., 2002, 2003). These researchers showed immobility stress caused more than double the amygdala remodeling than did unpredictable stress, and again in behavioral testing only the immobility stress increased anxiety-like behavior (Vyas et al., 2002). This research group followed this up with behavioral analysis of these two conditions (Vyas et al., 2004) and showed that 10 days of immobilization stress (2 h/a day) leads rodents to reach a ceiling in anxiety-like behavior while 10 days of unpredictable stress looks just like control. Besides showing an opposite stress-induced remodeling than hippocampal neurons, amygdala remodeling did not reverse 3 weeks after recovery from stress (Vyas et al., 2004). Joining forces with McEwen, Mitra et al. (Mitra et al., 2005) went on to show that even a single 2-h episode of immobility stress induced a delayed expansion in amygdala dendritic spines that paralleled a delayed development of generalized fear. The above findings converge on increased dendritic spines and/or branching on amygdala neurons as a candidate physical mechanism for the generalization of fear itself, which we discuss in more detail below.

However, to highlight the paradigm shift: the expectation up to this point was that repeated *unpredictable* stress induced the strongest behavioral change because of the inability to prepare oneself for what comes next, but unpredictability proved inferior to *inescapability*. Looking across these preclinical models, we would therefore predict that the degree of perceived "*entrapment*" will be a better predictor of amygdala enlargement in individuals exposed to ELA. A systematic review by Taylor et al. (2011) gathers extant evidence from behavioral studies and human studies of depression, psychosis, caregiver burden, chronic pain, anxiety, and traumatic stress that converges on a construct of perceived inescapability that defeat/helplessness contributes to and social support buffers against. Circling back to the "lived experiences" of ELA, when there is not literal entrapment, overwhelming health problems, legal problems, or financial issues may be emblematic of daily life and a form of figurative entrapment. This has potential major implications for poor mental health. Surveying clinical work on self-injurious behavior, elements of figurative entrapment played a role in 25–30% of suicides from 2003 to 2008 (Logan, 2011). Pivoting back to neurobiology, *dendritic expansion may be a compensatory effort to map environmental features to detect safety or avenues of escape*, but in these cases of entrapment, no safety can be achieved so the amygdala churns away, reaching a terminal toxic-metabolic shrinkage in the first decades of life.

A futile cycle attempting and failing to map unattainable safety could explain increased amygdala volume and activity after stress, while other limbic regions (hippocampus, PFC) demonstrate atrophy. We believe research in rodents and non-human primates suggest a testable model about the factors driving this enlargement. As McEwen detailed in multiple reports about allostatic load, the body increases certain functions to meet the demands of the stress, but long-term adaptation is costlier than true homeostasis. Connected to this, Ghosh and Chattarji (2015) examined recruitment of amygdala neurons specifically in the lateral nucleus during fear conditioning, and discovered not only that neurons tuned to a conditioned sound increased their activity after pairing with an aversive stimulus (mild shock), but also that neurons tuned to other sounds broadened their tuning to now respond to the conditioned stimulus. In other words, fear conditioning literally recruits a broader neural network in the amygdala, shifting the balance toward regional excitation. Importantly, a stronger aversive stimulus caused 30% of neurons tested to broadly generalize and respond to nearly any sound, a finding that could be recapitulated by artificially increasing neuronal excitability in the amygdala. A month later, Resnik and Paz (2015) published findings from electrical recordings throughout the three nuclei of the basolateral complex (basomedial, basolateral, and lateral, **Figure 2**) of macaques, and demonstrated that fear conditioning in primates follows the same pattern of not only strengthening neuronal signatures of environmental cues (sounds) present during an aversive stimulus (in this case a strongly aversive odor) and again broadening of the tuning of neurons not previously responding to sounds in the range of the conditioned stimulus. Just as in the rodent, *the degree of neuronal generalization matched the degree of behavioral fear generalization*. In short, primate and rodent amygdala expand the neural signatures not just of cues indicating danger but recruit more neurons to map the parameter space of similar cues. We believe this is part of a natural mechanism to find the bounds of danger and identify related signals of safety. In support of this, Amir et al. (2015) demonstrate that ∼70% of primary neurons and most interneurons in the rodent basolateral nucleus show a graded decrease in firing as the animals leave the safety of their nest and face a robotic predator, with 23% showing an opposite firing rate proportional to danger (possibly the analog of the minority of neurons that strengthened or generalized to cues). Therefore, a subset of amygdala neurons representing danger or generalized fear can drive recruitment of a broader population of amygdala neurons that map relative degrees of known safety.

It is as yet unclear if there is an equivalent fear generalization in primates proportional to the intensity of an aversive stimulus, but this is likely the mechanism by which individuals suffering ELA proportionally over-activate their amygdalae. We predict that this metabolically costly effort to map the environmental space surrounding an intense aversive event represents a key function of the basolateral amygdala as defining the boundaries of danger cues so that safety can be achieved. This is in line with imaging findings and neural network modeling that suggest greatest recruitment in situations of uncertain danger (Kim et al., 2003; Herry et al., 2007). But what if danger is inescapable in all conditions? If no associations with safety are found, and a stimulus is sufficiently intense, perpetual generalized fear responses may ultimately lead to overload and burnout of the "safety mapping" neurons of the amygdala as it endlessly pursues a spatiotemporal boundary to the threat.

CONNECTING AMYGDALA NEUROBIOLOGY TO DEVELOPMENTAL MAL/ADAPTATION AFTER ELA

If amygdala allostatic adaptations do indeed map to attempts to contextualize cues related to threat and vigilance, we believe there are clear developmental translations and connections between this neurobiological phenotype and the aberrant psychosocial and behavioral processes commonly seen after adversity [for a comprehensive review, see Cicchetti (2016)]. These include: disruptions with attachment, emotion processing, and social bonding. While allostatic load models have permeated aspects of developmental psychology, it will be important to increase crosstalk between these areas and to, as Cicchetti noted, "examine the prior sequences of adaptation or maladaptation in development that have contributed to a given outcome."

Related to attachment, forming a secure early bond provides an individual a base from which to explore and forge new experiences. At the least, this "stable base" literally provides a zone around a parent where "escape" through parental intervention can overcome any threat. Interestingly, individuals exposed to adversity often develop insecure attachment styles [see Cyr et al. (2010), for a meta-analysis], eliminating or at least destabilizing this zone of safety and leading to beliefs of others as unavailable or untrustworthy. This is strongly seen for individuals who have suffered maltreatment, but also for other types of ELA, including low household income, having a parent with a substance use issue, and lower maternal education. These changes could have profound implications for behavioral development, especially if individuals exposed to ELA believe that their caregivers are not safe enough supports for them to explore an environment. In the most extreme cases, infants with disorganized attachments will actually show freezing behavior toward caregivers. This strikingly parallels over-generalization in preclinical fear-conditioning work (Mahan and Ressler, 2012). As these individuals continue to develop, this "unsafe" representation may get expanded out to other individuals in their environments. Thinking about the usefulness of this expanded representation, if one is not sure of who signals safety and security (and what behavior is appropriate to execute in a context), it could be potentially "more adaptive" not to enact any behavior at all. Thinking about attachment and ELA, a number of interesting research findings may relate to aberrant contextual processing. For example, physically abused infants display higher rates of fearfulness, anger, and sadness during parent-infant interactions, compared to age-matched non-maltreated peers (Cicchetti and Ng, 2014). In contrast, neglected infants display blunted ranges of emotional expression, often with an increased duration of negative affect compared to non-maltreated infants. While clearly phenomenologically different, the aberrant processing of contextual safety could potentially explain each of these patterns. Abused infants may be over-contextualizing the negative affect that they experience with their mothers, while neglected infants may be uncertain how and under what circumstances they should be expressing positive emotions.

Moving forward in development and turning to emotion processing, aberrant understanding of contextual cues may connect to the alterations in threat sensitivity and hyper-vigilance commonly reported after ELA. As an illustration, a good deal of recent research, most notably by Pollak et al. (e.g., Pollak et al., 2000; Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003) finds an increased sensitivity to anger-related cues after abuse: with maltreated children perceiving angry faces as more salient relative to other emotions, display broader perceptual category boundaries for detecting anger, and require less visual information to perceive angry facial expressions. Interestingly, there is suggestive evidence that children exposed to poverty display similar (though subtler) biases toward threat. Indeed, children living in poverty tend to carefully monitor their environment for danger and maintain a low threshold for judging situations as threatening (Chen and Matthews, 2003; Chen and Paterson, 2006). Impoverished youth often exhibit larger cardiovascular responses than higher SES youth when confronted with ambiguous stimuli (Chen et al., 2004). These threat-bias results clearly represent differences in emotion processing but could also be seen as contextual over-generalization or lack of safety learning. Children exposed to adversity may be sensitized to threat, and then they believe threat (and fear) is the common and dominant (and potentially "default") emotion. In keeping with classic social-information processing work by Dodge et al. (1990), Dodge (1993), and Crick and Dodge (1994), if children exposed to ELA over-contextualize threat and perceive the world to be a hostile and unsafe place, they may respond with high-levels of aggression and potentially retaliatory violence (Connell and Goodman, 2002; Evans et al., 2008; Wilson et al., 2009; Jaffee, 2017; Peverill et al., 2020). The over-representation of subtle signs of threat are likely represented in the synapses and dendritic branching of the amygdala.

Connected to both attachment and emotion processing, those exposed to ELA often have problems with peer relations across multiple stages of development. Interestingly, different forms of ELA (e.g., maltreatment) are related to either withdrawal from or overt aggression toward peers. While many may consider these divergent phenotypes, both may be appropriate (and learned) responses to environmental entrapment and an inability to map safety signals. Youth either fight, attempting to escape perceived entrapment; or eventually surrender under the belief that breaking free is not possible. Furthermore, ELA-exposed youth often make errors in encoding social cues, exhibit biases toward attributing hostile intent, generate more aggressive responses, and positively evaluate aggression as an appropriate response (Teisl and Cicchetti, 2008). Over time, this may lead youth, especially who have suffered ELA and evince amygdala volumetric shrinkage, to select and structure later social interactions to recreate and *validate familiar relationship patterns as a means of reducing amygdala signals of uncertainty*. If the amygdala molecular machinery is also "burned out", it may be challenging to differentiate safety vs. threat. Indeed, adults exposed to ELA report greater interpersonal sensitivity, paranoia, and hostility (e.g., Liem and Boudewyn, 1999). Such patterns are most consistently noted in previously maltreated samples, but similar results have been reported in cumulative ELA (the "ACEs") with cumulative childhood trauma exposure by 16 years of age relating to negative social/peer outcomes (e.g., poorer quality of relationship with spouse/significant other and friends;

Copeland et al., 2018). Additional research indicates adults that suffer early social neglect are less likely to be married than their non-neglected peers (e.g., Tieman et al., 2005), and report fewer social supports and close confidants (Weiner and Kupermintz, 2001; Sigal et al., 2003). This suggests that overrepresentation of threat may come at the cost of learned social safety and affiliation mappings in networks connecting through the amygdala.

ENVIRONMENTAL MODERATION OF SAFETY LEARNING: INFLUENCE OF CAREGIVERS

While excitation-inhibition imbalances in the amygdala may be a consequence of ELA, the potential neural embedding of inescapability is not without counterweights. Breakthroughs in the last two decades have identified specific "*extinction cells*" in basolateral amygdala that receive inputs from portions of the prefrontal cortex (Herry et al., 2008; Likhtik et al., 2008; Strobel et al., 2015); in preclinical models, this includes cells in infralimbic (IL) PFC, with signals from this portion of PFC driving the Basomedial amygdala nucleus (sometimes termed "accessory basal nucleus") to inhibit fear by cues and contexts known to confer safety (Adhikari et al., 2015; Bloodgood et al., 2018). In primates, the evolutionary descendent of infralimbic PFC, subgenual cingulate or Brodmann Area 25 (BA25), was recently found to have a similarly heavy projection to the basomedial nucleus in macaques (Kim et al., 2018). Direct chemical overactivation broadly of BA25 in primates *increases* sympathetic tone, fear sensitivity and amygdala activation (Alexander et al., 2020), attesting to the ability of BA25 to also signal threat (likely via the paraventricular thalamus) and that broad overactivation favors fear over safety. Building on our model that the basolateral amygdala is recruited to build a finer mapping of the gradation from threat to safety, the BA25 signal may dampen fear-responding to known threats if previously learned escape mechanisms (e.g., stay away from the house when parent is intoxicated) are available. Conveniently, co-activation of infralimbic PFC and basolateral amygdala in rodents inhibits the ventral tegmental area (VTA), reducing the key dopamine signal that "unlocks" the basolateral complex (Marowsky et al., 2005; Patton et al., 2013).

This raises the possibility of a protective circuit (**Figure 5**) by which: (1) a novel stimulus triggers dopamine release from the VTA, leading to (2) activation of prefrontal and limbic regions including disinhibiting basolateral amygdala to alter the scope of fear, (3) safety and threat information is synthesized in subgenual BA25 and transmitted to primary cells in basolateral amygdala, which (4) may conclude the threat is manageable and deactivate the entire loop by turning off the VTA. Alternatively, sustained responses favor centromedial output nuclei, the site of greatest dopaminergic innervation in the human amygdala (García-Amado and Prensa, 2013), likely signaling innate and previously learned fears that flow through a BA25->paraventricular thalamus->centromedial amygdala circuit (Do-Monte et al., 2015; Penzo et al., 2015).

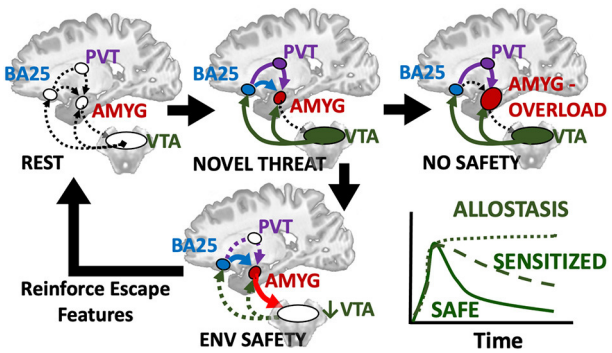

FIGURE 5 | Hypothesized loop by which a novel threat activates alerting/approach related ventral tegmental area (VTA) which provides dopaminergic innervation to prefrontal regions including the subgenual BA25 sends the resultant evaluation of situational safety through the uncinate fasciculus (blue) to basal and lateral amygdala nuclei and simultaneous threat signals through the paraventricular thalamus (PVT, purple) to the centromedial amygdala effectors. Co-activation of BA25 and amygdala safety circuits deactivates the VTA and cues encoding safety or escape are retained and reinforced as reward. If no safety is achievable, chronic activation of BA25 and amygdala including dopamine signals from the VTA lead to fear generalization or sensitization and favor the PVT relay and latent fears. A transient response or deactivation of dopamine appears to be a key manifestation of safety.

With this circuit in mind, we consider the groundbreaking work of Regina Sullivan, who showed in a rat model that there is a critical period during which associating a cue with a natural threat (e.g., fox odor) can paradoxically produce an *appetitive response* when conditioned in the presence of mother (Moriceau and Sullivan, 2006). It is unknown, as of yet, if there is an equivalent critical period in human development during which facing threatening situations in the safety of a parent causes reminders of the experience to induce positive and even antidepressant-like responses later in life (Rincón-Cortés et al., 2015). Sullivan's group traced this effect to a temporary drop in dopamine in the basolateral complex when the mother was present (Barr et al., 2009). More recently, her group found that developmental maturation of the infralimbic cortex->amygdala pathway supplants this effect (Robinson-Drummer et al., 2019), such that "social buffering" by mother beyond the critical period induces a negative correlation between amygdala and VTA activation, as measured with 2-deoxyglucose metabolic mapping (Opendak et al., 2019). In contrast, maternal maltreatment induced by resource scarcity closes the window such that maternal presence no longer deactivates amygdala within the critical period and at later ages maternal presence (perhaps appropriately) no longer deactivates the amygdala, nor does it produce the negative coupling of basolateral amygdala and VTA that would indicate convergent safety signals from BA25 and basomedial amygdala. While we are far from understanding all the components of this response, it builds on the model that the BA25-basolateral amygdala pathway likely carries signals of relative safety from a threat in the environment, consistent with human studies of structural connectivity (Tromp et al., 2019). Another recent human study suggests the major influence of parental warmth on subsequent

brain function and psychopathology in preadolescence, when this system comes online, is most evident in subgenual cingulate (BA25) activation and functional connectivity with amygdala (Butterfield et al., 2020). The neural substrate of positive parental and environmental influences that prevent psychopathology is likely BA25->basomedial amygdala circuit deactivation of VTA.

We can take this model one step further and consider how social buffering could lower the excitation:inhibition ratio of the amygdala so as not to rush experience-dependent plasticity during development. Zhang et al. (2020) mapped representations of aversive stimuli to a subtype of inhibitory cell in basolateral amygdala (expressing RSPO2) and safety signaling to a subtype expressing Dopamine Associated neuronal PhosphoProtein (DARPP-32; as known as Ppp1r1b). They then showed that direct reactivation in the absence of threat of the DARPP-32+ neurons that comprise the extinction (safety) memory trace produced a strong reward response in a contextual conditioning test. This raises the possibility that healthy amygdala development involves depositing layers of neurons, storing rewarding solutions to previously survived experiences and exposures that inhibit broad amygdala activation and fear generalization. It is not yet known whether these neurons are direct recipients of BA25 innervation or whether they differentially express dopamine receptors, but we speculate that social buffering in humans likely requires activation of the loop described above producing a pulse of dopamine and then a drop in basolateral amygdala dopamine and that this ultimately strengthens the DARPP-32 neurons in the basolateral complex.

Feedforward safety signals likely reduce the average excitation:inhibition ratio of the amygdala, permitting a slower maturation throughout adolescence and possibly more DARPP-32 cells that could activate positive affects in safe contexts. If an environment offers few to no safety signals, this maturation is accelerated according to the allostatic load model, progressing more rapidly to dendritic outgrowth, higher excitation:inhibition and an early allostatic peak that likely compresses stress buffering periods. It is unknown, however if the lack of any parental safety signals during this critical period, most exemplified by institutional neglect, leads to amygdala shrinkage in the absence of additional adversity. This is particularly challenging given the high incidence of further adversity in foster care and may be better studied in children with diffuse attachment disorder. We speculate that the lack of safety learning during critical periods leads to limited psychological and emotion regulation resources, represented by the safety learning possibilities wired through the DARPP-32+ cells, to overcome challenges. Lack of social safety learning likely leads to inability to detect and avoid dangerous environments and individuals, ultimately leading to similar outcomes.

Fortunately, future treatments may be able to mimic the temporal dynamics of dopamine signaling and repair or renew DARPP-32 cells in the basolateral complex, re-opening a window of plasticity for social safety learning. Consistent with this model, a single dose of the dopamine precursor L-DOPA/carbidopa facilitates fear extinction in rodents and humans through enhanced negative connectivity between ventromedial prefrontal regions and amygdala (Haaker et al., 2013). More recent work by Cisler et al. (2020) showed that L-DOPA/carbidopa enhances post-training reactivation of amygdala during memory consolidation, leading to enhanced extinction of fear in adults with PTSD. It is tempting to presume that this enhanced amygdala activation includes a preponderance of DARPP-32+ safety cells, but more research in this area is needed. It will be critical to integrate these findings into emerging theories about the neurobiological impacts of ELA, as well as models examining connections between ELA and psychopathology.

FURTHER CONSIDERATION OF ELA, AMYGDALA NEUROBIOLOGY, AND CLINICAL PRACTICE

As noted earlier, we took a more broad and inclusive definition of ELA here, but additional work is clearly needed to richly characterize and define stressful early life experiences. This work will come in many forms and will need to consider developmental context, "true" lived experiences, subjective perceptions, and the temporal dynamics of adversity exposure. Further cataloging of positive events and success overcoming challenges despite adversity will enrich our understanding of stress buffering and safety learning. Indeed, all of these factors will likely influence neurodevelopment and may impact trajectories of amygdala neurobiology.

First, and to be critical, many studies (e.g., Hair et al., 2015; Hanson et al., 2019) actually focus on developmental exposures, or the adverse contexts that youth develop in. This is in contrast to true experiences that a child *actually* encounters. To borrow an illustrative example from (McLaughlin et al., 2020), two children may be exposed to similar negative life circumstances (e.g., parental drug abuse), but experience very different things (i.e., parental hostility, vs. caregivers receiving drug treatment). This is very much the case for exposure to poverty and economic marginalization. Numerous studies have shown that poverty, an adverse exposure, is associated with a host of stressful experiences including: neighborhood violence, housing instability, and issues with household structure and organization (Evans and English, 2002). Illustrating this idea for child neglect, we think about the context of institutional rearing vs. parental neglect. Youth in institutional settings may actually have support from staff or peers within these congregate care settings (e.g., McCall et al., 2019) while those living with a neglectful parent may rarely feel safety and experience greater perceived entrapment. Clear measures of specific negative experiences, during adverse exposures, will surely aid in understanding the types and "dosing" of negative experiences likely to influence brain and behavior. In addition to this distinction, the field should expand assessments of the subjective perceptions of ELAs. Strong recent work by Danese and Widom (2020) underscores that participants' perceptions of their experience may be the most predictive of behavioral challenges. In a unique cohort of individuals followed since childhood with court-documented evidence of maltreatment and subjective reports of childhood maltreatment histories, these investigators found subjective reports of childhood maltreatment were more robust

predictors of psychopathology, regardless of whether objective (court) records substantiated maltreatment. Thinking more about exposures, experiences, and subjective perceptions may aid in clarifying inconsistent neurobiological findings.

Related to subjective perceptions, as well as the dichotomy of exposure vs. experience, dimensional models begin to overcome many of the limitations in past studies, but there is more work to do in this space especially related to developmental timing. For example, McLaughlin et al. (2014a), as well as Belsky et al. (2012) and Ellis et al. (2017), articulate potential dimensions of experience that may influence development (e.g., Deprivation vs. Threat; Harshness vs. Unpredictability); however, it is unclear if differences in developmental competencies, especially early in life, may cause the blurring of boundaries between ELA dimensions. For example, children exposed to early neglect while living in institutional care would, in theory, represent a "*deprivation*" ELA dimension. However, these children are often very young (<3 years of age) when they are in these settings, and the global neglect they are experiencing may impinge upon attachment processes (represented in basomedial and superficial nuclei, **Figure 3**). Such experiences, perhaps due to subjective perceptions and processes, would then actually be threatening in nature—the lack of a clear attachment figure would cause heightened vigilance to environmental dangers. This actually fits well with results from Tottenham's group that has found post-institutionalized children who suffered early social neglect have alterations in the amygdala, both structurally and functionally (e.g., Cohen et al., 2013; VanTieghem et al., 2021) and more recently found amygdala volume predicted later stress hormone responses (VanTieghem et al., 2021). Of important note, we believe compelling distinctions exist in non-human animal models of stress for inescapable vs. unpredictable stressors, but believe it still too early to synthesize these ideas to the exposure vs. experience distinction in humans. We are hopeful that future conceptual work could continue progress in this space.

Furthermore, while research teams often catalog many specific occurrences of stress, many types of common (*day-to-day*) experiences may not rise to the level of a "formal ELA." For example, there is a litany of research on "expressed emotion" and risk for poor mental health (Butzlaff and Hooley, 1998; Weintraub et al., 2017). Expressed emotion is hostility, criticalness, and excessive involvement of family members toward someone in the family with identified mental health problems (Weintraub et al., 2017). These family relational patterns can represent a psychosocial stressor that interacts with individuals' diatheses, eventually culminating in relapse (Hooley and Gotlib, 2000). Put another way—growing up with a parent suffering from substance use disorder, or who is hostile, may mean unpredictable bursts of anger or threatening behavior; this may instill a chronic stressful alertness, or influence attachment processes, as youth look for any sign of a bad mood or known aggravating factor. Clinically, research has found that hostility and emotional over-involvement slowed progress with interventions such as exposure therapy (Tarrier et al., 1999). However, and connected back to our conceptualization of the amygdala, hostile and emotionally boundaryless contexts may be perceived as psychologically unsafe and, ultimately, an inescapable stressor. It will be important to think about this and related elements of normal and atypical parenting in relation to neurobiology [for thoughtful review in this space, see Farber et al. (2020)].

Forging connections between these elements of adversity, our neurobehavioral conceptualization of the amygdala, and clinical outcomes, high or chronic levels of ELA, or ELA coupled with recent stressors, may eventually embed perceptions or feelings of inescapability and "being trapped." In many cases, this may take multiple psychosocial forms, from legal and financial problems to chronic pain, to anxiety, depression, and other forms of psychopathology. Amygdala neurobiological changes (e.g., initial dendritic expansion; hyper-excitability) may be compensatory efforts to map environmental features to detect safety in the context of these stressors; however, with high-levels of psychosocial burden and/or limited social support (limited formation of DARPP-32+ escape solutions), no safety can be achieved so the amygdala churns away, reaching a terminal toxic-metabolic shrinkage. These psychosocial perceptions and neurobiological changes may indeed explain many "Deaths of Despair" in populations exposed to ELA (Bohnert and Ilgen, 2019). Declining opportunity or inability to escape deleterious life circumstances cause many to turn to opioids or other drugs to cope. In extreme cases (and exposure to multiple ELAs), this may lead to extreme learned helplessness and suicidality. This broad conjecture fits well with extant data showing elevated drug use and abuse, as well as suicide attempts in many exposed to ELAs (Dube et al., 2001, 2003; Brodsky and Stanley, 2008). For example, work has found that ∼30% of suicide attempts among women and 23% of those among men were attributable to having experienced repeated ELAs (e.g., physical abuse, sexual abuse, witnessing domestic violence; Afifi et al., 2008). Clinicians working with individuals with high ELA may be able to interrupt this deleterious cycle by helping clients see potential "escapes" out of multiple/compounding, real (or perceived) psychosocial challenges.

THINKING ABOUT STIGMA, RESILIENCE, AND "HIDDEN TALENTS"

While we believe that the model that we advance here is critical to understanding the sequalae of ELA, it is also important to acknowledge the balancing act that researchers and connected groups are intending to strike in thinking about the impact of ELAs. First, we are mindful of the potential stigma in connecting neurobiology to experiences of trauma, poverty, and other adversities. Without realizing it, one can create what many have termed "*biosocial determinism*" and the unintended, neurobiological rationalization of adversity-related disparities (Pitts-Taylor, 2019). Our aims are, instead, that leveraging ours and connected ideas related to the neurobiology of ELA can not only promote prevention, but also push larger structural changes at institutional levels to reform potential societal factors correlated with ELAs. For example, scientific research on institutionalization, and the child neglect common to many of these congregate care settings, has spurred many policy-driven changes aimed at transforming child protective services and bolstering support for families and communities (Llorente et al.,

2003; McCall et al., 2013; Berens and Nelson, 2015). Second, there is the additional connected risk of implicitly disparaging low-income households. Poverty and experiencing economic marginalization do not produce child maltreatment, and abuse occurs across all strata of the socioeconomic spectrum. Similarly, poverty is not synonymous with stress; however, poverty is associated with experiencing greater numbers of stressful life events and other hazards to youth development (Evans and English, 2002). We are mindful that parents and caregivers are often seen as responsible for these elements, while systemic factors contributing to adversity, such as social inequality and racism, routinely receive less attention and focus. We are encouraged by recent perspectives emerging from science and technology studies pushing for the transformation of research on ELA in how it is absorbed and discussed in different social and policy circles (Müller and Kenney, 2020).

Of additional note, neurobiological methods can homogenize people who have suffered ELA and often obscure the variety of strengths present within individuals. There is a growing literature on "*hidden talents*" that underscores many individuals growing up in high-adversity context have intact, or even enhanced, social, cognitive, and affective skills (Ellis et al., 2017, 2021; Frankenhuis et al., 2020). For example, economically marginalized adults often show enhanced procedural learning (i.e., acquiring stimulus–response associations) compared to their higher socioeconomic status peers. Scholars in this area, notably Ellis and Frankenhuis, have underscored that differences in neurobiology or behavior may be useful adaptations in responses to the adverse contexts; these differences may promote adaptive functioning, as one thinks about the different contexts that individuals inhabit. Relatedly, there is a large and well-developed body of research on resilience to ELA (Masten et al., 1999; Southwick et al., 2014), as well as post-traumatic growth (Tedeschi and Calhoun, 2004). This is outside the scope of our review, but at the broadest level, these bodies of research underscore most individuals who suffer ELA do not experience mental or physical health disorders. Negative outcomes, like higher aggression or greater depression, are enhanced, but these are not deterministic links. Many factors (e.g., social support, cognitive functioning) protect youth in the context of severe adversity (for thorough discussion, see the work of Ann Masten, e.g., Masten et al., 2021), We, as a field must be mindful that: (1) many exposed to ELA do not evince significant impairments; (2) there may be positive change that occurs as a result of the struggle with highly challenging life crises (Infurna and Jayawickreme, 2019); and (3) successful outcomes may be achieved through differential trajectories of neural and behavioral functioning. Holding these collective concepts in mind can limit the further marginalization of ELA-exposed populations and will no doubt aid in supporting youth exposed to adversity.

SPECIFIC FUTURE DIRECTIONS MOTIVATED BY OUR CONCEPTUAL MODEL

The innovative and interesting conceptual framework put forth here suggests a number of critical future directions in the study of ELA and amygdala neurobiology. These include formal tests of the proposed model, to considered revisions of some preclinical stress manipulations. We elaborate on these below.

First, and related to our model, there is the potential to adapt and deploy magnetic resonance spectroscopy (MRS) to probe markers of amygdala neurobiology, including cell density, membrane phosphocholines, second messenger turnover, and other critical molecular markers. The second author and his colleagues at University of Wisconsin-Madison (e.g., Nacewicz et al., 2012) have been able to measure pooled glutamate and glutamine to probe potential excitotoxicity in typical and atypical samples (e.g., Individuals with Autism). This work involves custom fitting of MRS voxel to amygdala anatomy as we found an extraordinary sensitivity to partial inclusion of different amygdala nuclei. Moving forward, although capturing the average neurochemical concentrations of an entire amygdala is spatially imprecise, the temporal and neurochemical precision offered by emergent functional spectroscopy techniques promises to unravel the exact dynamics of excitatory glutamate, inhibitory GABA and even the acetylcholine (Bell et al., 2019), which is enriched in the basolateral nucleus. Additionally, novel diffusion weighted techniques (NODDI; Zhang et al., 2012) estimate neurite (dendritic) complexity in gray matter with a resolution approaching structural anatomical images, and could longitudinally characterize the normal vs. allostatic peak amygdala volumes. Complementing this, the novel PET tracer UCB-J binds the synaptic marker SV2A as a direct, albeit relative, measure of synapse density (Finnema et al., 2016). Combining these methods, longitudinal increases or decreases in synapses and dendritic trees can be combined with glutamate and GABA measurements to determine the relative outgrowth of inhibitory vs. excitatory synapses, thereby directly tracking development of allostatic load. These tools can help screen for environmental influences and treatments that halt the amygdala overgrowth or at least the toxic shrinkage.

Second, and related to stress manipulations in preclinical work, there are many experimental design changes that could shed light on the full scope of neurobiological consequences of ELA. To be blunt, few, if any, rodent models are developmentally sensitive (cf. work by Regina Sullivan et al.). The seminal studies by Vyas et al. motivated a host of human studies on ELA; however, this work was completed in young adult rodents. Additional work that is often less highlighted, by Rosenkranz et al. completed similar stress manipulations and found many critical differences (e.g., number of spontaneously firing neurons vs. firing rates) in animals exposed to stress as juveniles vs. in adulthood. Little work, in our opinion, goes far enough to truly understand the true developmental impact of ELA. In line with recommendations from Callaghan et al. (2019), it is and will be critical to consider the developmental ecology and "goals" of an organism (e.g., attachment; independence) in thinking about neurodevelopment. Such ideas also connect to a translational challenge in most preclinical studies—nearly all of these paradigms do not adequately capture the transactional nature of stress (Lazarus and Folkman, 1987). To be concrete, in humans, ELA (e.g., the multiple stressors associated with poverty; maltreatment) may affect how an individual responds to

subsequent life stressors (Monroe and Harkness, 2005; Hammen, 2018).

In particular, we believe it will be important to consider the interaction of ELAs with stressors during childhood and also later in life. ELA may affect how individuals respond to subsequent life stressors and heighten risk for poor mental health (Monroe and Harkness, 2005; Hammen, 2018). Rich support has been found for this "stress sensitization" or "two-hit models," for example, women with exposure to one or more childhood adversities (e.g., family violence, parental psychopathology) were more likely to become depressed by a lower "dose" of total stress than women without such adversity (Hammen et al., 2000). This is true for depression, anxiety, and PTSD, and found during childhood, adolescence, and adulthood (Dougherty et al., 2004; Kendler et al., 2004; Harkness et al., 2006; Espejo et al., 2007; McLaughlin et al., 2010). Framed in terms of our safety-mapping model: avoiding a known threat (guided by BA25-basomedial amygdala signals), such as avoiding a parent or home at the slightest sign of a bad mood, reduces the proportion of one's life environment in which an individual feels safe. A subsequent adverse event in another environment thought to be "safe," e.g., escaping an abusive home and ending up in a romantic partnership that involves further abuse, quickly exhausts the system and hastens the allostatic changes. We also note that a single act of random unprovoked violence can sometimes prove more difficult to treat than repeated predictable abuse from a known individual, again likely representing the challenge of finding anything other than temporal signs of safety. In this way, ELA may sensitize individuals to later challenges whereby mild to moderate stressors complete a mapping of inescapability, intensifying fear generalization by glucocorticoid-induced amygdala remodeling. Put more clearly—the occurrence of ELA may fundamentally change how an organism moves through and approaches the world. There may be changes in coping, self-concept, and other complex psychosocial processes that then interact with later events. Limited neurobiological work has examined such ideas (cf., Hanson et al., 2015a, 2018, 2019), but it would be a fruitful target for future investigations.

Third, a hopeful discovery by Sara et al. two decades ago (Przybyslawski et al., 1999) suggested that conditioned fear becomes labile when reactivated and its reconsolidation can be disrupted such that the stored fear was no longer susceptible to stress-induced reinstatement. Put simply, we can erase fear conditioning by combining reactivation and pharmacotherapy. While much had to be learned in the process, which was traced to synapse-specific protein synthesis triggered by calcium influx primarily from NMDA receptors (Nader et al., 2000; Debiec et al., 2006), it ultimately yielded promising candidates for medication-assisted exposure therapy. This emerging work has identified NMDA-antagonistic agents (D-cycloserine, ketamine; Guastella et al., 2008; Kalisch et al., 2009; Otto et al., 2010; Das et al., 2019), a mitochondrial enhancer (methylene blue; Telch et al., 2014; Zoellner et al., 2017) and the anti-adrenergic propranolol as having potential to erase the fight-or-flight component of fear learning (Kindt et al., 2009; Soeter and Kindt, 2015; Brunet et al., 2018). Several caveats were discovered including: (1) the memories must be reactivated or the treatment adds nothing (2) the medication exposure must end well, with relative safety achieved by the end or treatment could worsen the fear and (3) conveniently, the medication need not be administered prior to the exposure but in a 4–6 h window of neuroplasticity after treatment (4) in the case of propranolol a much higher dose is required (40 mg acute dose) than would typically be prescribed for situational phobia. Armed with these tools, we may be able to eliminate the biological fear response and accelerate treatment (Brunet et al., 2018), but interestingly subjective fear can lag the biology significantly (Rothbaum et al., 2014; Soeter and Kindt, 2015). This opens an unprecedented avenue to understand the separate components of biological fear generalization and subjective impressions of entrapment. Further, as noted above, other agents such as L-DOPA hold the promise of enhancing the extinction memory rather than the disrupting the fear memory itself. Given that activation of extinction neurons in the absence of fear neurons is rewarding in rodents, head-to-head comparisons of different medication-assisted therapies could disentangle the relative contributions of safety signals and fear signals in the allostatic load model. More importantly, our limited mental health resources may be able to increase efficiency for treating the enormous toll of ELA and adult mental illness currently inflicted by the COVID-19 pandemic.

Finally, it will be important to investigate more deeply potential "tipping points" in human ELA exposed samples and whether cascades related to ELA may be pernicious and excitotoxic. Adversity may potentially start at (and continue into) multiple, different stages of development. In addition, there may be psychosocial consequences of ELA that may increase the likelihood of volumetric shrinkage in exposed individuals. Such a developmental incorporation may have important insights for the prediction, prevention, and treatment of negative outcomes related to ELA. ELA may cause psychosocial (or neurobiological) alterations at one time point, but individuals who have suffered these adversities keep engaging in, as well as creating, different experiences. Outcomes at later time points may be related to this initial adaptation, but also could be due to the interaction of early changes and current situational experiences. Clinically, those working with adversity-exposed populations often know that previous (negative) adaptations in their clients may create later residue that individuals who have suffered bring to the different situations that subsequently greet them. Connected to the ideas we advance above, future work will need to consider not just age and duration of a stressor, but also the "escapability" or proportion of contexts affected (e.g., daycare, home, and a relative's house). Thoughtful execution of these multiple ideas, across preclinical and human studies, could significantly advance our understanding of the neurobiological sequelae of ELA, the mediating connections between ELA and psychopathology, and more basic science questions such as nature vs. nurture.

CONCLUDING REMARKS

Here, we put forward an integrated model of amygdala neurodevelopment to think about inconsistencies in research on ELA, as well as the behavioral consequences of adversity. We must all continue to dissect heterogeneity, think about theoretical integrations of stress neurobiology and developmental psychology, and clarify complex relationships between ELA

and related long-term mental health challenges. We are excited to pursue many of the future directions we proposed here and would be excited about improvements in preclinical and human studies focused on early stress exposure. Continued progress in these spaces, potentially guided by the theoretical model laid out here could be particularly important for predicting, preventing, and treating the consequences of ELA.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://github.com/jlhanson5/Hanson_Nacewicz_Frontiers_Amygdala_Review_Data.

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AUTHOR CONTRIBUTIONS

Both authors contributed equally to the outlining, drafting, and writing of the manuscript.

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Influence of *FKBP5* Variants and Childhood Trauma on Brain Volume in Non-clinical Individuals

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The present study aimed to investigate the possible influence of childhood trauma and its interaction effect with 10 single-nucleotide polymorphisms (SNPs) of the FK506-binding protein 51 (*FKBP5*) gene on brain volume in non-clinical individuals. One hundred forty-four non-clinical volunteers (44 men and 100 women) were genotyped with respect to 10 variants (rs9296158, rs3800373, rs1360780, rs9470080, rs4713916, rs4713919, rs6902321, rs56311918, rs3798345, and rs9380528) of *FKBP5*. Participants underwent magnetic resonance imaging (MRI) scan and psychological assessments such as the childhood Trauma Questionnaire (CTQ), Hospital Anxiety and Depression Scale, rumination response scale, and quality of life assessment instrument. Individuals with the high CTQ score showed enlarged volume of the left orbitofrontal cortex (OFC) if they have childhood trauma-susceptible genotype of *FKBP5* rs3800373, rs1360780, rs4713916, rs4713919, rs6902321, and rs3798345 and enlarged volume of the left middle temporal gyrus (MTG) if they have childhood trauma-susceptible genotype of *FKBP5* rs3800373, rs1360780, rs4713916, and rs3798345. Among those with the childhood trauma-susceptible genotype, the left OFC and left MTG showed significant negative correlations with positive feelings about life, and the left OFC showed significant positive correlations with negative cognition. This is one of the few studies to identify the volume alteration of the left OFC and the left MTG for the *FKBP5* gene–childhood trauma interaction in non-clinical individuals.

Keywords: *FKBP5*, childhood trauma, orbitofrontal cortex (OFC), middle temporal gyrus (MTG), neuroplastic compensatory mechanism

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INTRODUCTION

The neurobiological mechanisms, where genes and psychosocial environments dynamically interact with each other, influence subsequent brain functions, structures, and psychopathologies (Hyde et al., 2011; Hyde, 2015; Kim et al., 2018). The presence of both genetic variants with risky allele and childhood adversity may synergistically increase the risk of psychopathology (Kim et al., 2018). It has been also reported that individuals with both genetic variants with risk allele and childhood adversity were associated with volume alteration in several regions

(Carballedo et al., 2013; Harrisberger et al., 2015). In contrast, those risks have not been observed among individuals with the same gene allele and exposure to childhood adversity (Marusak et al., 2016). Such a difference may be participants' ethnicity and population differences (González-Castro et al., 2017). However, the mixed results related to the genotype effect were revealed in the same ethnicity (Jin et al., 2019).

Exposure to emotional and physical adversity during childhood has been associated with poor mental and physical health, academic performance, and socio-occupational functioning (McCrory et al., 2011). Childhood trauma could lead to neuroanatomical changes in highly vulnerable regions of the brain (McCrory et al., 2011), which include the amygdala, hippocampus, prefrontal cortex (PFC), visual and auditory cortices, and thalamus (Teicher et al., 2016). Furthermore, studies have identified the effects of childhood adversity on neural correlates of emotional, reward, and cognitive processing, which are commonly associated with the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC; Hart and Rubia, 2012). According to a recent meta-analysis, individuals exposed to childhood trauma showed distinctive differences in brain volumes when compared to controls: smaller superior temporal gyrus, insula, parahippocampal gyrus and middle temporal gyrus (MTG), and enlarged right superior frontal and left middle occipital gyri (Lim et al., 2014).

Previous studies have reported the relationship between genetic variants and greater susceptibility to psychopathological development in individuals with childhood trauma (Caspi et al., 2002; Binder et al., 2008; Bradley et al., 2008; Weder et al., 2009; Hornung and Heim, 2014). According to the genetic susceptibility model, genetic variations reflect differential sensitivity to environmental factors through the stress response system accompanied by the hypothalamic–pituitary–adrenal (HPA) axis (de Castro-Catala et al., 2017). Stress-response endophenotypes indicate higher dexamethasone suppression test levels or higher odds of psychiatric diagnosis. For their connections to cortisol-induced responses, they have been associated with several neuronal functions and brain structures regarding mood regulation, particularly in the context of childhood trauma (Gerritsen et al., 2017). The FK506-binding protein 51 (*FKBP5*), which is involved in transcriptional regulation of the HPA axis, is one of the major candidate genes (White et al., 2012). *FKBP5* protein of the *FKBP5* gene, which is located on chromosome 6p21 (Binder, 2009), is involved in regulating glucocorticoid receptor activity and providing a negative feedback loop in the HPA axis (Han et al., 2017). The overexpression of this protein can reduce the hormone-binding affinity and nuclear translocation of the glucocorticoid receptor, downregulating the expression of anti-inflammatory proteins in neuronal nuclei (Wochnik et al., 2005). Several studies pointed out that the *FKBP5* gene, with impact of childhood trauma, can predict brain structural alterations (Grabe et al., 2010; White et al., 2012; Fani et al., 2013; Pagliaccio et al., 2014; Holz et al., 2015).

Although the studies to date are difficult to compare, we have identified the inconsistency in previous studies. Some studies have narrowed their focus solely on direct *FKBP5* genetic effects on brain alteration, without factoring in an individual's life stress (Zobel et al., 2010; Fani et al., 2013; Fujii et al., 2014). Other studies have looked at both the main and interaction effects of *FKBP5* but considered only one or a few single-nucleotide polymorphisms (SNPs) of *FKBP5* (Holz et al., 2015; Grabe et al., 2016; Tozzi et al., 2016). Since they have used a few SNPs in their study, effects of other SNPs in *FKBP5* and childhood trauma exposure on structural alteration in brain could not be fully identified. Furthermore, studies have used discrete variables in childhood trauma exposure (Grabe et al., 2016), which may fail to capture the original characteristics of the continuous scale. Also, to include the total score of childhood trauma scale to measure the effect of childhood trauma experiences seems a more comprehensive approach.

To overcome these inconsistencies, the present study explored multiple *FKBP5* SNPs and their interaction with the environment on brain volume alteration. We included a total of 10 *FKBP5* SNPs (rs9296158, rs3800373, rs1360780, rs9470080, rs4713916, rs4713919, rs6902321, rs56311918, rs3798345, and rs9380528) to investigate their possible interaction effect with childhood trauma. These 10 SNPs have been located in intron 7, Bin3 (site 6) which were differentially methylated in response to childhood trauma in the presence of the *FKBP5* risk allele (Klengel et al., 2013; Yehuda et al., 2016). Some SNPs have been reported as relevant predictors of stress susceptibility and risk factors of psychiatric symptoms (Binder et al., 2008; White et al., 2012; Zannas et al., 2015). Furthermore, the continuous scale was used to assess effects of total childhood trauma.

Given the previous findings, examining SNPs related to methylation at intron 7 of *FKBP5* in the effects of childhood trauma is a cogent approach. The *FKBP5* risk allele carrier and early trauma exposure lead to demethylation of intron 7 CpGs in *FKBP5*, which further amplifies genotype-dependent differences (Klengel et al., 2013). Genetic differences lead to divergent chromatin conformations and interactions of long-range enhancers with the transcription start site. It is related to a differential transcriptional activation of *FKBP5* by glucocorticoid receptor activation depending on childhood trauma. These changes in chromatin structure increased cortisol levels and thus glucocorticoid receptor binding, leading to changes in DNA methylation in intron 7, further increasing the differential responsiveness of *FKBP5* to glucocorticoid receptor activation (Klengel et al., 2013). Holocaust survivors and their offspring have methylation changes on the same site in a functional intronic region of the *FKBP5* gene (Yehuda et al., 2016). These effects were observed at bin 3/site 6 intron 7. According to these findings in two studies, it seems to be important to examine SNPs in intron 7 of *FKBP5* in the context of trauma effects.

We hypothesized that the influence of childhood trauma on brain volume might differ depending on the *FKBP5* genotype in non-clinical individuals. Gene and psychosocial environmental interaction effects might be influential to the brain regions. Such effects may play a critical role in predicting physiological and behavioral adaptations to stress including modulation of the HPA axis (Binder et al., 2008; Roy et al., 2010; Tatro et al., 2010; Zou et al., 2010; Supriyanto et al., 2011; Bevilacqua et al., 2012).

MATERIALS AND METHODS

Participants

A total of 161 Korean non-clinical volunteers who lived in Seoul city and Gyeonggi province were initially included in the present study. They were recruited from the local community through flyers and posters. Participants with any history of neurological or other mental diseases were excluded from the study through the initial screening interviews, which were based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. They were also excluded if they had shown any abnormal brain imaging findings. All participants had no history of psychiatric medical treatment and were not in need of any clinical mental health service/advice. Therefore, seven participants were excluded due to missing data from psychological variables; six additional participants were omitted from the analyses due to missing *FKBP5* gene data; and four participants who missed magnetic resonance imaging (MRI) and found abnormal MRI image were also excluded. A final sample of 144 non-clinical volunteers (44 men and 100 women) with a mean age of 46.93 ± 13.37 (years) was included. Study protocols were approved by the Institutional Review Board at Inje University Ilsan Paik Hospital (IRB no. 2015-07-025), and the study was conducted according to the Declaration of Helsinki. All participants provided a written informed consent prior to the study enrollment.

Psychological Measures

Childhood Trauma Questionnaire

The Korean validated version of the Childhood Trauma Questionnaire (CTQ) was used to assess participants' childhood trauma (Yu et al., 2009). The CTQ consists of five subscales of various childhood traumas, including emotional abuse, physical and sexual abuse, and emotional and physical neglect, and another scale for detecting minimization and denial. It is known as a useful self-report questionnaire in eliciting retrospective reports of childhood maltreatment from young adults (Everson et al., 2008). The CTQ consists of 28 items and is rated with a 5-point Likert scale ranging from 1 ("never true") to 5 ("very often true"). Higher scores indicate more traumatic experiences in childhood. The coefficient alpha of the CTQ was 0.77 in the current study.

Hospital Anxiety and Depression Scale

The Korean-validated version of the Hospital Anxiety and Depression Scale (HADS) was used to assess anxiety and depression symptoms (Oh et al., 1999). It is a self-reported questionnaire with seven items for describing anxiety and with seven items for describing depression. It is assessed with a four-point Likert scale, ranging from 0 (no problems) to 3 (maximum distress). Higher scores indicate higher levels of anxiety and depression. The coefficients of each subtype of HADS were 0.87 (anxiety) and 0.78 (depression) in the current study.

Ruminative Response Scale

The Korean-validated version of the Ruminative Response Scale (RRS) was conducted to assess ruminative responses (Kim et al., 2010). It is comprised of three subscales specifically evaluating self-reproach, contemplation, and depressive rumination. It is a self-reported questionnaire with 22 items and is rated using a four-point Likert scale ranging from 1 ("almost never") to 4 ("almost always"). Higher scores indicate higher levels of rumination responses. The coefficient alpha of the RRS was 0.94, and those of each subscale of the RRS were 0.86 (rumination), 0.89 (contemplation), and 0.91 (depressive rumination) in the current study.

World Health Organization Quality of Life Assessment Instrument

The Korean validated and abbreviated version of World Health Organization Quality of Life assessment instrument (WHOQOL) was used to assess the quality of life (Min et al., 2000). It consists of five subscales including physical health, psychological health, social relationships, environment, and general health. It is a self-reported questionnaire with 26 items and is rated using a 4-point Likert scale ranging from 1 ("almost never") to 4 ("almost always"). Higher scores indicate higher levels of quality of life. The coefficient of the WHOQOL was 0.89 in the current study.

Genetic Data

All participants had their blood sampled to extract DNA using a NanoDrop ND-1000 UV-Vis Spectrophotometer. Genomic DNA was then diluted to a 5-ng/μL concentration in 96-well polymerase chain reaction (PCR) plates. TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, United States) were obtained, and the probes were labeled with FAM or VIC dye at the 5′ end and a minor-groove binder and non-fluorescent quencher at the 3′ end. PCR was done in 5 μL of a mixture containing 2 μL of a DNA sample, 0.125 μL of each TaqMan SNP Genotyping Assay (Thermo Fisher Scientific), 2.5 μL of TaqMan Genotyping Master Mix (Thermo Fisher Scientific), and 0.375 μL of distilled water. Amplification and detection were done with a detection system (QuantStudio 12K Flex Real-Time PCR System, Thermo Fisher Scientific) with the profile of 50°C for 2 min and 95°C for 10 min, followed by 60 cycles of 95°C for 15 s and 6°C for 1 min. After the PCR amplification, allelic discrimination was performed using the same machines (QuantStudio 12K Flex Real-Time PCR System), which was considered an endpoint plate read. The QuantStudio 12K Flex SOFTWARE calculated the fluorescence measurements made during the plate read and plotted Rn values based on the signals from each well. Finally, the analyzed plates were used to perform automatic or manual allele calls.

There were three positive samples and one negative control sample for each plate, and we confirmed positive controls with a clustering image. Our intra-genomic DNA samples of known genotypes were used for positive control. Ten *FKBP5* SNPs (rs9296158, rs3800373, rs1360780, rs9470080, rs4713916, rs4713919, rs6902321, rs56311918, rs3798345, and rs9380528) were genotyped to calculate ancestral proportions for all study participants. We calculated genotype frequencies for each individual polymorphism and evaluated the Hardy–Weinberg equilibrium to check the data quality and genotype error. The chi-squared test was used to compare the observed numbers of each

genotype with those expected for the population following chi-squares distribution with one degree of freedom (Weir, 1996). All statistical tests and visualization of differentially expressed genes were conducted using R version 3.3.3.

In line with our study hypothesis and previous studies (Binder et al., 2008; Yun et al., 2020), all participants were divided into two genotype groups by allele frequencies (**Table 1**).

MRI Acquisition

Prior to MRI scanning, participants passed an MRI safety check and obtained a detailed explanation of the imaging protocol. Participants underwent MRI examination on a 1.5-T Magnetom Avanto (Siemens, Erlangen, Germany). To minimize the head motion, the manufacturer placed foam pads against each side of the head. A high-resolution T1-weighted MRI volume dataset was acquired (227 × 384 acquisition matrix, a 210 × 250 field-of-view, 0.9 × 0.7 × 1.2 voxel size, a total of 87,168 voxels, an echo time (TE) of 3.42 ms, a repetition time (TR) of 1,900 ms, slice thickness of 1.2 mm, and a flip angle of 15°).

The voxel-based volumetric analysis was conducted using computational Anatomy Toolbox 12 (developed by Christian Gaser, University of Jena)[1] with the Statistical Parametric Mapping (SPM) 12 software package (Wellcome Department of Cognitive Neurology, London, United Kingdom) (Ashburner and Friston, 2005; Ashburner, 2007). Given the differences in the morphology of East Asian and Caucasian brains, the structural T1 images were registered to an ICBM East Asian template. Spatial normalization of the images was generated using the DARTEL algorithm (Ashburner, 2007). The images were then segmented into gray matter, white matter, and cerebrospinal fluid (Ashburner and Friston, 2005). Jacobian-transformed tissue probability maps were used to correct gray matter segments for volume differences. The volume of the regions was extracted using the Neuromorphometrics atlas, available in SPM 12, provided by Neuromorphometrics, Inc[2].

Regions of Interest

Fourteen regions of interest (i.e., seven regions of both hemispheres) were as follows: Several MRI studies have shown the interaction between *FKBP5* SNPs and the childhood trauma and how the structural and functional alterations of the hippocampus and amygdala (White et al., 2012; Pagliaccio et al., 2014; Holz et al., 2015) heavily relate with it. Likewise, volume alterations in the insula, superior and middle temporal gyrus, and anterior cingulate cortex were identified from abused individuals with the TT genotype of the *FKBP5* rs1360780 (Grabe et al., 2016). Other studies reported regions associated with both childhood trauma and *FKBP5* variants were amygdala,

[1]http://dbm.neuro.uni-jena.de/cat

[2]http://neuromorphometrics.com

TABLE 1 | Demographics, psychological measures, and *FKBP5* variants of participants (*N* = 144).

	***Mean* (*SD*) or *N* (%)**			***N* (%)**
Demographics		***FKBP5* genotype frequencies**		
Men	44 (30.6%)	rs9296158	GG	69 (47.90)
Women	100 (69.4%)		AG + AA	75 (52.10)
Age [yrs]	46.93(13.37)	rs3800373	AA	86 (59.70)
Education [yrs]	13.68(3.05)		AC + CC	58 (40.30)
Psychological measures		rs1360780	CC	84 (58.30)
CTQ	42.60(15.15)		CT + TT	60 (41.70)
Emotional Neglect	10.74(5.45)	rs9470080	CC	69 (47.90)
Physical Abuse	8.47(4.49)		CT + TT	75 (52.10)
Sexual Abuse	6.67(2.79)	rs4713916	GG	90 (62.50)
Emotional Abuse	8.05(4.24)		AG + AA	54 (37.50)
Physical Neglect	8.68(3.49)	rs4713919	GG	79 (54.90)
HADS-anxiety	6.45(3.70)		AG + AA	65 (45.10)
HADS-depression	6.89(3.64)	rs6902321	TT	74 (51.40)
RRS	39.81(12.25)		CT + CC	70 (48.60)
Rumination	14.01(4.25)	rs56311918	TT	101 (70.10)
Contemplation	12.34(4.78)		CT + CC	43 (29.90)
Depressive Rumination	13.47(5.20)	rs3798345	CC	93 (64.60)
WHOQOL	81.45(12.39)		CT + TT	51 (35.40)
Physical Health	23.54(4.40)	rs9380528	AA	110 (76.40)
Psychological Health	18.39(3.18)		AG + GG	34 (23.60)
Social Relationships	9.76(2.05)			
Environment	25.74(4.92)			
General health	6.55(1.37)			

CTQ, Childhood Trauma Questionnaire; HADS, Hospital Anxiety and Depression Scale; RRS, Ruminative Response Scale; WHOQOL, World Health Organization Quality of Life assessment instrument.

hippocampus, and OFC (Hart and Rubia, 2012; Fani et al., 2013; Pagliaccio et al., 2014; Holz et al., 2015; Han et al., 2017).

Statistical Analysis

The genotypic distributions of the 10 SNPs located in *FKBP5* were evaluated by the Hardy–Weinberg Equilibrium. Normality for all psychological variables was tested using the skewness and kurtosis. Skewness less than 2 and kurtosis less than 7 were considered to be moderately normally distributed (Curran et al., 1996). All variables in our results were within the range of normal distribution. After checking for normality, Pearson's correlation analysis with bootstrapping at a 5,000-sampling rate was performed. Following the correlation analysis, a regression analysis using Macro PROCESS for SPSS (version 2.16.3) was performed to examine the moderation effect of *FKBP5* variants on the relationship between childhood trauma and brain volume. CTQ was considered as a predictor, *FKBP5* variants were considered as moderators, and outcomes were considered as volume of brain regions. Age, sex, years of education, and total intracranial volume (TIV) were controlled as covariates. Anxiety and depression scale were also controlled, since a few participants who were enrolled had high scores in anxiety and depression scale. False discovery rate (FDR; Benjamini and Hochberg, 1995) with an adjusted $p < 0.10$ was used to correct for multiple comparisons. We used a significance level of 0.10 to alleviate excessive control.

Subsequently, the Johnson–Neyman technique (Hayes, 2013) was applied to probe an interaction effect. The Johnson–Neyman technique aligns the moderation variable in a continuous manner and computes the regions of significance for interactions by examining the significance between the predictor and outcome variables.

Finally, a partial correlation analysis was conducted on the brain regions where the moderation effect occurred to examine their relationship with psychological variables. The results were divided among each genotype of SNPs for comparison. Age, sex, years of education, TIV, anxiety, and depression scale were controlled as covariates. Five thousand bootstrapped samples were generated (Westfall, 2011). All statistical analyses were performed using SPSS version 21 (IBM Inc., Chicago, IL, United States).

RESULTS

Descriptive Statistics

Demographics, psychological measures, and the genotype frequencies of the 10 *FKBP5* SNPs of participants are presented in **Table 1**. The genotypic distributions of the 10 SNPs located in *FKBP5* did not deviate from the Hardy–Weinberg Equilibrium ($p > 0.05$) (Zintzaras, 2010) except for rs9380528, which was excluded from the analysis. Demographics and psychological measures between genotypes of each SNP are shown in **Supplementary Table 1**. There were no significant differences in demographics and psychological measures between genotypes of *FKBP5* variants except for sex differences of rs1360780 and rs56311918.

Moderation Effect

Moderation effects which were statistically significant after applying the FDR correction for multiple comparisons ($p < 0.10$) are shown in **Table 2**. Therefore, six significant moderation effects of *FKBP5* variants on the relationship between CTQ and the left OFC were identified (**Figure 1** and **Supplementary Table 2**). More specifically, the moderation model was significant in *FKBP5* rs3800373 ($R^2 = 0.591$, $p < 0.001$), rs1360780 ($R^2 = 0.584$, $p < 0.001$), rs4713916 ($R^2 = 0.581$, $p < 0.001$), rs4713919 ($R^2 = 0.582$, $p < 0.001$), rs6902321 ($R^2 = 0.762$, $p < 0.001$), and rs3798345 ($R^2 = 0.586$, $p < 0.001$). The main effects of CTQ and interaction effects between CTQ and *FKBP5* variants were significant in these six moderation models.

Furthermore, four significant moderation effects of *FKBP5* variants on the relationship between CTQ and the left MTG were identified after FDR correction (**Figure 1** and **Supplementary Table 3**). More specifically, the moderation model was significant in *FKBP5* rs3800373 ($R^2 = 0.697$, $p < 0.001$), rs1360780 ($R^2 = 0.694$, $p < 0.001$), rs4713916 ($R^2 = 0.692$, $p < 0.001$), and rs3798345 ($R^2 = 0.696$, $p < 0.001$). The main effects of CTQ

TABLE 2 | Statistically significant interaction effects of childhood trauma and *FKBP5* variants on alteration of brain volumes ($N = 144$).

	L orbitofrontal cortex			R orbitofrontal cortex			L middle temporal gyrus			R middle temporal gyrus		
Interaction effect	***B***	***P***	P_{FDR}	***B***	***P***	P_{FDR}	***B***	***P***	P_{FDR}	***B***	***P***	P_{FDR}
CTQ × rs9296158	0.0078	0.0113	0.1017	0.0061	0.0435	0.2097	0.0258	0.043	0.2097	0.0275	0.0136	0.1142
CTQ × rs3800373	0.0110	0.0008	**0.0784**	0.0076	0.0204	0.1506	0.0391	0.0040	**0.0784**	0.0309	0.0097	0.1017
CTQ × rs1360780	0.0098	0.0027	**0.0784**	0.0062	0.0577	0.2507	0.0369	0.0067	**0.0844**	0.0291	0.0150	0.1181
CTQ × rs9470080	0.0078	0.0103	0.1017	0.0060	0.0432	0.2097	0.0285	0.0239	0.1506	0.0242	0.0282	0.1692
CTQ × rs4713916	0.0092	0.0044	**0.0784**	0.0051	0.1153	0.4332.	0.0376	0.0054	**0.0784**	0.0248	0.0362	0.1983
CTQ × rs4713919	0.0088	0.0041	**0.0784**	0.0046	0.1341	0.4828	0.0290	0.0239	0.1506	0.0197	0.0794	0.3227
CTQ × rs6902321	0.0084	0.0056	**0.0784**	0.0065	0.0309	0.1770	0.0325	0.0107	0.1017	0.0252	0.0233	0.1506
CTQ × rs56311918	0.0024	0.5846	0.8682	0.0021	0.6239	0.8735	0.0179	0.3159	0.8180	0.0308	0.0465	0.2100
CTQ × rs3798345	0.0106	0.0018	**0.0784**	0.0054	0.1169	4332	0.0410	0.0038	**0.0784**	0.0248	0.0466	0.2097

P_{FDR} values after FDR correction (alpha < 0.10) were listed at the third line for multiple comparisons (i.e., 7ROIs × 2unilateral × 9SNPs). We present only CTQ-by-FKBP5 variant interactions corresponding to brain regions where P_{FDR} was significant.

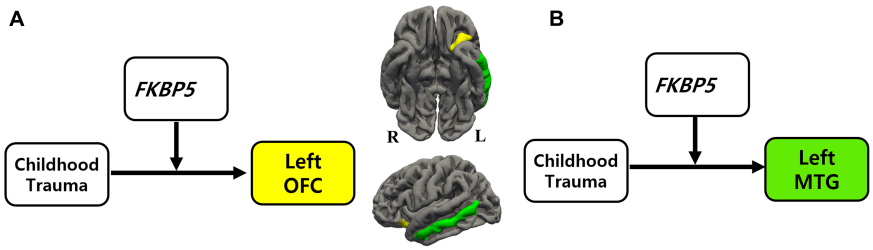

FIGURE 1 | The moderation effect of *FKBP5* variants on the relationship between childhood trauma and brain volume. [(a)]Survived six moderation effects of *FKBP5* variants (including rs3800373, rs1360780, rs4713916, rs4713919, rs6902321, and rs3798345) after FDR correction (alpha < 0.10) in the left OFC. [(b)]Survived four moderation effects of *FKBP5* variants (including rs3800373, rs1360780, rs4713916, and rs3798345) after FDR correction (alpha < 0.10) in the left MTG. *FKBP5*, FK506-binding protein 5; OFC, orbitofrontal cortex; MTG, middle temporal gyrus.

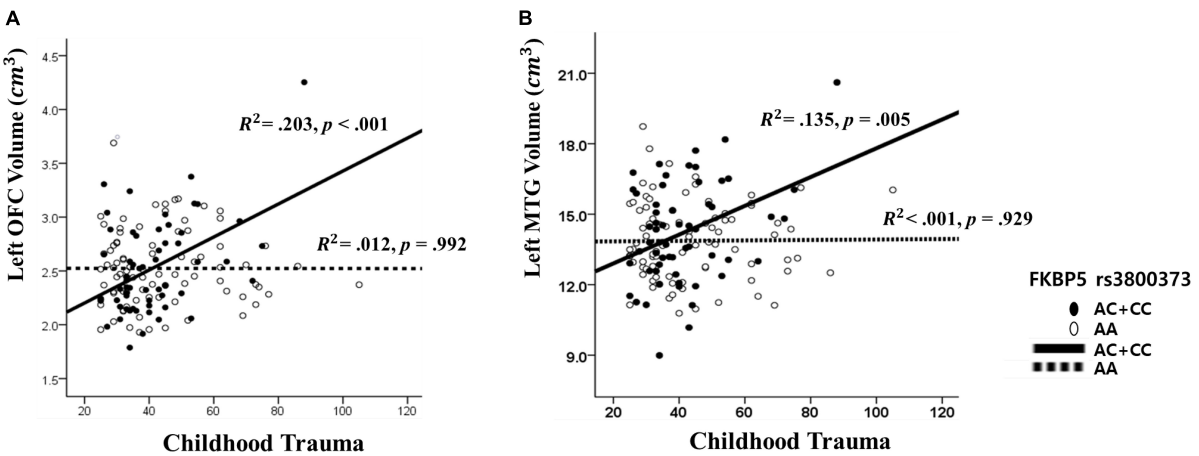

FIGURE 2 | The interaction effect of *FKBP5* rs3800373 and childhood trauma on **(A)** the left orbitofrontal cortex and **(B)** the left Middle Temporal Gyrus. **(A)** The other interaction effects had similar patterns in rs1360780, rs4713916, rs4713919, rs6902321, and rs3798345. **(B)** The other interaction effects had similar patterns in rs1360780, rs4713916, and rs3798345.

and interaction effects between CTQ and *FKBP5* variants were significant in these four moderation models.

Probing an Interaction

The results from The Johnson–Neyman analysis indicated that in rs3800373, the positive relationship between CTQ and the left OFC volume was significant in those with AC + CC genotype (B = 0.011, t = 3.628, p < 0.001) (**Figure 2**). As for rs1360780, the positive relationship between CTQ and the left OFC volume was significant in those with the CT + TT genotype (B = 0.010, t = 3.628, p < 0.001). As for rs4713916, the positive relationship between CTQ and the left OFC volume was significant in those with the AG + AA genotype (B = 0.009, t = 3.494, p = 0.001). As for rs4713919, the positive relationship between CTQ and the left OFC volume was significant in those with the AG + AA genotype (B = 0.009, t = 3.539, p = 0.001). As for rs6902321, the positive relationship between CTQ and the left OFC volume was significant in those with the CT + CC genotype (B = 0.008, t = 3.450, p = 0.001). As for rs3798345, the positive relationship between CTQ and the left OFC volume was significant in those with CT + TT genotype (B = 0.001, t = 3.749, p = 0.001) (**Supplementary Table 4**).

Furthermore, as for rs3800373, the positive relationship between CTQ and the left MTG volume was significant in those with the AC + CC genotype (B = 0.042, t = 3.744, p < 0.001) (**Figure 2**). As for rs1360780, the positive relationship between CTQ and the left MTG volume was significant in those with the CT + TT genotype (B = 0.041, t = 3.628, p < 0.001). As for rs4713916, the positive relationship between CTQ and the left MTG volume was significant in those with the AG + AA genotype (B = 0.041, t = 3.666, p < 0.001). As for rs3798345, the positive relationship between CTQ and the left MTG volume was significant in those with the CT + TT genotype (B = 0.045, t = 3.728, p < 0.001) (**Supplementary Table 4**).

Correlation Analysis

The left OFC was positively correlated with depressive rumination in those with the AC + CC genotype of rs3800373 (r = 0.314, p = 0.025), CT + TT genotype of rs1360780 (r = 0.320, p = 0.016), AG + AA genotype of rs4713916 (r = 0.366, p = 0.011), AG + AA genotype of rs4713919 (r = 0.259, p = 0.049), CT + CC genotype of rs6902321 (r = 0.307, p = 0.015), and CT + TT genotype of rs3798345

TABLE 3 | Brain regions affected by the interaction between *FKBP5* variants and the childhood trauma and the related psychological variables among genotypes

***r* with left OFC**		**Depressive rumination**	**Physical health**		**Psychological health**
rs3800373	AC + CC	**0.314 (*p* = 0.025)**	**−0.298 (*p* = 0.033)**		−0.240 (*p* = 0.090)
	AA	0.077 (*p* = 0.499)	−0.052 (*p* = 0.650)		−0.121 (*p* = 0.286)
rs1360780	CT + TT	**0.320 (*p* = 0.019)**	**−0.278 (*p* = 0.044)**		−0.265 (*p* = 0.055)
	CC	0.064 (*p* = 0.586)	−0.060 (*p* = 0.605)		−0.016 (*p* = 0.891)
rs4713916	AG + AA	**0.366 (*p* = 0.011)**	**−0.351 (*p* = 0.015)**		−0.283 (*p* = 0.054)
	GG	0.067 (*p* = 0.549)	−0.010 (*p* = 0.927)		0.015 (*p* = 0.893)
rs4713919	AG + AA	**0.259 (*p* = 0.049)**	−0.243 (*p* = 0.066)		−0.246 (*p* = 0.063)
	GG	0.125 (*p* = 0.298)	−0.011 (*p* = 0.925)		0.025 (*p* = 0.834)
rs6902321	CT + CC	**0.307 (*p* = 0.014)**	**−0.264 (*p* = 0.037)**		**−309 (*p* = 0.014)**
	TT	0.028 (*p* = 0.819)	−0.051 (*p* = 0.678)		0.025 (*p* = 0.842)
rs3798345	CT + TT	**0.365 (*p* = 0.015)**	**−0.302 (*p* = 0.047)**		**−0.316 (*p* = 0.037)**
	CC	0.056 (*p* = 0.609)	−0.044 (*p* = 0.689)		−0.026 (*p* = 0.813)
***r* with left MTG**		**Physical health**	**Psychological health**	**Social relationship**	**Quality of life**
rs3800373	AC + CC	−0.267 (*p* = 0.059)	−0.265 (*p* = 0.060)	**−0.277 (*p* = 0.049)**	**−0.295 (*p* = 0.035)**
	AA	0.079 (*p* = 0.486)	0.175 (*p* = 0.122)	−0.055 (*p* = 0.626)	0.103 (*p* = 0.363)
rs1360780	CT + TT	−0.245 (*p* = 0.077)	**−0.286 (*p* = 0.038)**	−0.239 (*p* = 0.084)	**−0.280 (*p* = 0.035)**
	CC	0.083 (*p* = 0.472)	0.222 (*p* = 0.050)	−0.056 (*p* = 0.627)	0.132 (*p* = 0.250)
rs4713916	AG + AA	**−0.330 (*p* = 0.023)**	**−0.296 (*p* = 0.044)**	−0.265 (*p* = 0.071)	−0.258 (*p* = 0.080)
	GG	0.089 (*p* = 0.419)	0.168 (*p* = 0.128)	−0.026 (*p* = 0.816)	0.089 (*p* = 0.421)
rs3798345	CT + TT	−0.258 (*p* = 0.091)	**−0.314 (*p* = 0.038)**	−0.291 (*p* = 0.056)	−0.281 (*p* = 0.054)
	CC	0.048 (*p* = 0.658)	0.139 (*p* = 0.199)	−0.033 (*p* = 0.762)	0.071 (*p* = 0.516)

Controlling for the effects of age, sex, years of education, TIV, anxiety and depression scale. The generation of 5000 bootstrapped samples.
OFC, orbitofrontal cortex; MTG, middle temporal gyrus.

(r = 0.365, p = 0.015). Also, the left OFC was negatively correlated with physical health in those with the AC + CC genotype of rs3800373 (r = −0.298, p = 0.033), CT + TT genotype of rs1360780 (r = −0.278, p = 0.044), AG + AA genotype of rs4713916 (r = −0.351, p = 0.015), CT + CC genotype of rs6902321 (r = −0.264, p = 0.037), and CT + TT genotype of rs3798345 (r = −0.302, p = 0.047). Furthermore, the left OFC was negatively correlated with psychological health in those with the CT + CC genotype of rs6902321 (r = −0.309, p = 0.014) and CT + TT genotype of rs3798345 (r = −0.316, p = 0.037). In contrast, these correlations were not identified in those with the other genotype of *FKBP5* variants (**Table 3**).

The left MTG was negatively correlated with physical health in those with the AG + AA genotype of rs4713916 (r = −0.330, p = 0.023). The left MTG was negatively correlated with psychological health in those with the CT + TT genotype of rs1360780 (r = −0.286, p = 0.038), AG + AA genotype of rs4713916 (r = −0.296, p = 0.044), and CT + TT genotype of rs3798345 (r = −0.314, p = 0.038). The left MTG was negatively correlated with social relationship in those with the AC + CC genotype of rs3800373 (r = −0.277, p = 0.049). The left MTG was negatively correlated with quality of life in those with the AC + CC genotype of rs3800373 (r = −0.295, p = 0.035) and CT + TT genotype of rs1360780 (r = −0.280, p = 0.041). In contrast, these correlations were not identified in those with the other genotype of *FKBP5* variants (**Table 3**).

DISCUSSION

The present study aimed to explore the relationship among childhood trauma, 10 SNPs of *FKBP5*, and brain volume in non-clinical individuals. Our major findings were as follows. First, interaction of *FKBP5* variants and childhood trauma predicted volume alteration of the left OFC and left MTG. Second, individuals with the childhood trauma-susceptible genotype of *FKBP5* variants showed an enlarged volume of the left OFC and left MTG when childhood trauma was higher. Third, volume alteration of the left OFC and the left MTG showed significant negative correlations with positive feeling about life, and the left OFC showed significant positive correlations with negative cognition.

We found that the interaction of *FKBP5* and childhood trauma predicted volume alteration of the left OFC and left MTG in non-clinical individuals. A previous study reported that exposure to early life trauma has been shown to weaken developing brain structure and permanently set the stress response system on high alert (University CotDCaH, 2009). Furthermore, a genetic predisposition which is modulated or triggered by environmental factors could be a significant influencing factor on behavior and emotion (Saveanu and Nemeroff, 2012; Mandelli and Serretti, 2013). The *FKBP5* gene is an outstanding candidate that supports the model of stress-induced HPA-axis dysregulation in which genetically susceptible subjects are considered as long-standing biological risk (Grabe et al., 2016).

In our study, individuals with the childhood trauma-susceptible genotype of *FKBP5* variants showed enlarged volume of the left OFC when childhood trauma was higher. More specifically, the *FKBP5* genotype ("AC + CC" genotype of rs3800373, "CT + TT" genotype of rs1360780, "AG + AA" genotype of rs4713916, "AG + AA" genotype of rs4713919, CT + CC genotype of rs6902321, and "CT + TT" genotype of rs3798345) expressed an enlarged volume of the left OFC if they have shown higher scores of childhood trauma. Individuals with these six genotypes seemed to be susceptible to childhood trauma-related change of the left OFC according to a genetic vulnerability-stress theory (Caspi et al., 2003; Eley et al., 2004). In contrast, individuals with the other genotypes seemed to show no effect on brain volumetric change interacted by childhood trauma. Furthermore, enlarged volumes of the left MTG were found in the "AC + CC" genotype of rs3800373, "CT + TT" genotype of rs1360780, "AG + AA" genotype of rs4713916, and "CT + TT" genotype of rs3798345. Similarly, individuals with these three genotypes seemed to be susceptible to childhood trauma. In contrast, individuals with the other genotypes seemed to show no effect on brain volumetric change for childhood trauma.

Risk and protective genotype (or allele) of *FKBP5* are not well defined. For example, the rs3800373 "C" carriers appear to confer risk for stress-related disorders, whereas the "AA" genotype appears to be non-risk (Binder et al., 2008). In our study, the enlarged volumes of left OFC and left MTG were observed in those with the "C" carriers in the case of higher childhood traumatic experience. A specific genotype could not be conceptualized as neither inherently "good" nor "bad" (Halldorsdottir and Binder, 2017). It would be better to understand this genotype as having more responsivity (plasticity) or less responsivity (static) to environmental stress (Belsky et al., 2009). Our findings suggest that this susceptibility may also be determined by the severity of childhood traumatic experience.

Our findings suggested that enlarged volumes of the left OFC and the left MTG could imply neuroplastic compensatory mechanisms for protecting against environmental stress. In AC + CC genotypes, the volume of the left OFC showed significant positive correlations with negative cognition (e.g., depressive rumination) and negative correlations with positive feeling about life (e.g., physical health, psychological health). Previously, it was repeatedly reported that the OFC is important for adapting behavior about the emotional value of cues (Schoenbaum and Roesch, 2005; Roberts, 2006; Abler et al., 2009), cognitive evaluation of the environment, and expression of appropriate emotion-related responses (Rempel-Clower, 2007). OFC damage is associated with impaired control abilities (Hebscher et al., 2016) and neurotoxic effects in those related to pathological anxiety (de Wit et al., 2014). An extensive meta-analysis comprising 331 individuals with a history of childhood trauma and psychiatric comorbidities showed deficits in the OFC (Lim et al., 2014).

In susceptible-childhood trauma genotypes (i.e., "AC + CC" genotype of rs3800373, "CT + TT" genotype of rs1360780, "AG + AA" genotype of rs4713916, and "CT + TT" genotype of rs3798345), the volume of the left MTG was negatively correlated with positive feeling about life (e.g., physical health, psychological health, social relationship & quality of life). Previously, it was reported that the MTG is important for emotional memory processing, deductive reasoning, and recognition of emotional faces (Goel et al., 1998; Acheson and Hagoort, 2013; Meng et al., 2016). Thus, youth group who experienced physical abuse before the age of 12 (Lim et al., 2018) and adolescents with callous-unemotional conduct problems (De Brito et al., 2009) had greater middle temporal cortical volume than healthy controls. Yang et al. (2017) reported an enlarged volume of the left MTG linked to childhood traumas in first-episode major depressive disorder. The enlarged volume of the MTG in the non-chronic depression group and youth group can be a compensatory regulation for the early stage psychopathology of depression (Yang et al., 2017; Lim et al., 2018). In this context, our results showing enlarged volumes of the left OFC and the left MTG might be related to overworking as a function of compensatory cognitions (Brooks et al., 2016), which may prevent pathological anxiety and psychiatric symptoms.

Despite the significant findings reported, some limitations are present in this study. First, the current study is a cross-sectional design which presents a difficulty to disentangle whether alteration of the brain volume was present prior to the childhood trauma (McLaughlin et al., 2015). Second, we have a multiple testing issue for the correlation analysis between brain volumes and psychological variables.

In conclusion, this is one of the first studies to examine the brain structural effects of the gene–childhood trauma interaction with *FKBP5* variants in non-clinical individuals. Second, individuals with the childhood trauma-susceptible genotype of *FKBP5* variants showed enlarged volume of the left OFC and the left MTG when childhood trauma was higher. Our results suggested that enlarged volumes of the left OFC and the left MTG seem to imply a neuroplastic compensatory function of the brain protecting against environmental stresses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB no. 2015-07-025. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AK performed the methodology, formal analysis, project administration, and writing of the original draft. SK and HJ performed the material preparation, data collection, and software

analysis. HL performed the visualization and english language editing. S-HL performed the funding acquisition, investigation, review, and supervision. All authors contributed to the study conception and design, discussed the results and commented on the manuscript at all stages, and approved the final manuscript.

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Sex-Specific Associations Between Trauma Exposure, Pubertal Timing, and Anxiety in Black Children

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Recent research has linked early life stress (ELS), such as trauma exposure, with early puberty. Early puberty has also been identified as a risk factor for poor mental health outcomes. However, these two paths have primarily been examined independently. In addition, more studies have examined these associations in girls than boys, and findings for boys remain mixed. We hypothesized that early puberty (relative to peers) would be positively associated with both prior trauma exposure and concurrent anxiety symptoms. We anticipated that these associations might differ by sex. We tested these hypotheses within a cross-sectional sample of 133 8- to 13-year-old Black girls and boys with trauma exposure. The association between trauma and accelerated pubertal timing was sex-specific: it was positive for girls and negative for boys. We stratified subsequent analyses by sex. Regression analyses indicated that early puberty relative to peers predicted more anxiety symptoms for girls but not boys, after accounting for trauma exposure. A statistical mediation analysis indicated that, for girls, the positive association between trauma exposure and anxiety was partially mediated by pubertal timing. These results indicate that trauma exposure may have sex-specific effects on pubertal timing and anxiety risk in Black children. We also found that, for girls, trauma may increase risk for adverse outcomes by prompting earlier puberty, which is linked to higher anxiety. These findings are consistent with cascading effects of trauma across development, and highlight the need for further study of sex-specific mechanisms.

Keywords: puberty, trauma, development, sex differences, anxiety, mental health, Black Americans

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INTRODUCTION

Early puberty is a transdiagnostic risk factor for adverse mental health outcomes (Hamlat et al., 2019; Sumner et al., 2019; Colich et al., 2020a). Multiple forms of early life stress (ELS), including harsh caregiving (Belsky et al., 2010), low socioeconomic status (Braithwaite et al., 2009; Gur et al., 2019), traumatic events (Sumner et al., 2019), and sexual abuse (Negriff et al., 2015; Noll et al., 2017) have been associated with earlier puberty (Henrichs et al., 2014). Many studies have examined associations between ELS and pubertal timing

(e.g., Henrichs et al., 2014), or between pubertal timing and mental health (Ullsperger and Nikolas, 2017). However, few studies have concurrently examined how ELS, pubertal timing, and mental health outcomes are related (but see e.g., Sumner et al., 2019; Colich et al., 2020a), making it unclear whether ELS-linked early puberty is independent of or a pathway through which ELS impacts mental health (Copeland et al., 2019). Elucidating how these factors are associated is relevant to understanding mechanisms through which ELS impacts developmental processes and mental health (Joos et al., 2018).

Although puberty is ubiquitous, its developmental timing, physiology, and associated neuroendocrine changes are sex-specific (Grumbach and Styne, 2003; Shirtcliff et al., 2009). Adrenarche, the first phase of puberty (Merikangas et al., 2010; Kessler et al., 2012), begins around 6–8 years of age in girls and approximately 1 year later in boys (Vijayakumar et al., 2018). Gonadarche, the second phase, also begins earlier in girls than boys and is driven by increasing estradiol and progesterone in girls but by testosterone in boys (Grumbach and Styne, 2003; Shirtcliff et al., 2009). These gonadal hormones are linked to sex differences in neural development (Herting et al., 2014), emotion processing (Mueller et al., 2014), and behavior (Peper and Dahl, 2013). Together, the changes driven by puberty contribute to a shift from pre-pubertal gender parity in the prevalence of most internalizing psychopathologies to an approximately 2:1 prevalence in girls vs. boys during adolescence (Merikangas et al., 2010).

Pubertal development can be characterized in multiple ways. A common approach is to assess pubertal development at one time point relative to an objective scale such as the Tanner Stages. Individuals' pubertal development can then be characterized in terms of a stage, e.g., Tanner Stage 3. Comparison of individuals' pubertal development relative to their peers is referred to as *pubertal timing* (e.g., Mendle et al., 2010; Ellis et al., 2011; Marceau et al., 2011; Horvath et al., 2019). Another approach is to quantify the rate at which puberty unfolds, for instance, the time elapsed between early vs. late puberty. This is referred to as *pubertal tempo* (e.g., Mendle et al., 2010; Ellis et al., 2011; Marceau et al., 2011; Horvath et al., 2019). Assessments of tempo require longitudinal data that can compare development within an individual across time.

Regardless of whether pubertal timing or tempo is measured, most studies analyze measurements of pubertal timing or tempo from individuals relative to other factors of interest, such as ELS. Assessments of pubertal timing within specific populations, such as Black adolescents who live in an urban context, facilitates comparison of individuals' pubertal timing relative to same-age peers. Because many characteristics and experiences of the cohort are similar, this facilitates examination of how exposures that vary, like trauma exposure, may impact pubertal timing. This approach may be particularly advantageous for study populations that are typically underrepresented in developmental research, such as Black children with trauma exposure, because it characterizes an individual's development relative to other individuals from the same population. Recently, this approach has been used widely in studies that examine developmental trajectories, including in studies of pubertal timing and accelerated aging (see e.g., Jovanovic et al., 2017; Gur et al., 2019; Colich et al., 2020b; Bittner et al., 2021; Herzberg et al., 2021).

More studies of pubertal development have examined associations between different types of ELS and pubertal timing in girls than in boys (Belsky et al., 2010; Henrichs et al., 2014; Marshall, 2016; Mendle et al., 2016; Noll et al., 2017; Copeland et al., 2019; Sear et al., 2019). For girls, results consistently indicate that ELS is linked to earlier pubertal timing, whereas findings are more mixed for boys. Some studies report ELS-linked early pubertal timingfor girls but not boys (James et al., 2012), whereas others report similar positive associations for both (Gur et al., 2019; Sumner et al., 2019). Some evidence suggests that for boys ELS may accelerate how quickly puberty progresses (tempo) rather than when puberty begins (timing; Negriff et al., 2015). Several studies have found opposite associations between ELS and puberty in girls vs. boys, such that ELS predicts earlier pubertal timing in girls but slower timing for boys (Semiz et al., 2009; Johnson et al., 2018; Suglia et al., 2020). Overall, fewer studies have examined associations between ELS and puberty in boys, and studies that include boys have yielded mixed results.

As with studies of ELS and pubertal timing, more studies of associations between pubertal timing and mental health have focused on girls than on boys (Copeland et al., 2019; Colich et al., 2020a). Overall, similar patterns have been reported in the associations between pubertal timing and psychopathology for girls and boys (Ullsperger and Nikolas, 2017), but see also Marceau et al. (2011). A recent meta-analysis reported small but significant positive associations between pubertal timing and psychopathology in girls and boys (Ullsperger and Nikolas, 2017). Results from a large study of White boys and girls reported mixed patterns of sex differences in the associations between pubertal timing, tempo, internalizing symptoms, and externalizing symptoms (Marceau et al., 2011). Earlier timing and tempo were positively associated with internalizing symptoms for girls but not boys. For boys, faster tempo of pubic hair and genital development was associated with more externalizing symptoms. For girls, earlier timing and faster tempo were both positively associated with externalizing. In contrast, another study that included children from multiple racial and ethnic groups reported that both earlier pubertal timing and faster tempo predicted more depressive symptoms in boys, but that tempo was a more robust predictor than timing (Mendle et al., 2010). Girls' depressive symptoms were elevated for those with early timing, but tempo was not predictive (Mendle et al., 2010). A more complete understanding of how different aspects of pubertal timing impact psychopathology in diverse populations may help to explain the emergence of sex differences in the prevalence of internalizing psychopathologies during puberty.

Trauma exposure, an extreme form of ELS, impacts many aspects of development (Mandelli et al., 2015; Baumeister et al., 2016; Op den Kelder et al., 2018; Gur et al., 2019). There is ongoing debate regarding the definitions

and classification of ELS and adversity in the literature (see e.g., Nelson and Gabard-Durnam, 2020), and a summary of the varied uses of these terms is beyond the scope of the present work, which is focused on the effects of trauma. The Diagnostic and Statistical Manual of Mental Disorders (5th Ed., American Psychiatric Association, 2013) defines trauma as:

> *"Exposure to actual or threatened death, serious injury, or sexual violence in one (or more) of the following ways: directly experiencing the traumatic event(s); witnessing, in person, the traumatic event(s) as it occurred to others; learning that the traumatic event(s) occurred to a close family member or close friend (in case of actual or threatened death of a family member or friend, the event(s) must have been violent or accidental); or experiencing repeated or extreme exposure to aversive details of the traumatic event(s) (p. 271)."*

Traumas that align with this definition are referred to as Criterion A traumas. Importantly, an individual's self-identified race may impact what events are considered Criterion A traumas. For instance, interactions with police may be more likely to constitute Criterion A traumas for individuals who do not identify as White. Trauma exposure is associated with elevated risk for developing anxiety disorders (e.g., Leen-Feldner et al., 2008; Copeland et al., 2014).

Recent studies suggest that, in addition to trauma's direct negative effects on mental health, it also increases risk for accelerated puberty timing. This acceleration of pubertal timing in turn further increases risk for psychopathology (Colich et al., 2020a). Together, these would constitute a double blow of trauma exposure on mental health risk *via* direct effects and indirect effects through pubertal timing. Interestingly, one recent study found that childhood trauma exposure predicted accelerated pubertal timing similarly for girls and boys, but that pubertal timing did not predict internalizing or externalizing psychopathology for either group (Sumner et al., 2019). This result contrasts with multiple prior studies (Ullsperger and Nikolas, 2017), and highlights the need to examine the consistency of these associations are across different types of ELS and trauma exposures.

Both ELS broadly, and childhood trauma exposure specifically, are prevalent (Fairbank and Fairbank, 2009; Sacks and Murphey, 2018; Copeland et al., 2019; Merrick et al., 2019), making it critical to identify pathways through which they impact development and mental health outcomes. More population-level research has examined the effects of ELS generally than childhood trauma specifically in the context of health outcomes (Merrick et al., 2019), and there is an outstanding need to understand the specific effects of trauma on developmental processes. There is substantial evidence that Black Americans experience higher levels of both trauma (Gillespie et al., 2009) and ELS (Sacks and Murphey, 2018) than other groups within the United States (Merrick et al., 2019). However, Black children are relatively understudied in research on ELS and/or trauma and pubertal acceleration.

Our study examines associations between trauma exposure, pubertal timing, and anxiety symptoms within a cross-sectional sample of 8- to 13-year-old Black children who are at elevated risk for psychopathology due to trauma exposure and low socioeconomic status (SES). Prior studies with this cohort have identified associations between trauma exposure and both biomarkers and symptoms of anxiety (Jovanovic et al., 2014; Stenson et al., 2021), therefore we chose to focus on this aspect of mental health. We hypothesized that ELS would be associated with accelerated pubertal timing for girls (e.g., Colich et al., 2020a), but given the mixed findings in boys we anticipated that this association might differ for boys (Semiz et al., 2009; Kogan et al., 2015; Johnson et al., 2018; Suglia et al., 2020). We also hypothesized that pubertal timing would be positively associated with anxiety symptoms given prior findings from multiple populations (e.g., Ullsperger and Nikolas, 2017) and from this cohort (Jovanovic et al., 2014; Stenson et al., 2021), and that this association might be stronger for girls than boys. Finally, given recent evidence that pubertal timing statistically mediated the association between ELS and psychopathology (Colich et al., 2020a) we anticipated that if our first two hypotheses were supported, that pubertal acceleration would partially statistically mediate the association between trauma and anxiety symptoms for girls.

MATERIALS AND METHODS

Children (N = 133, 64 girls) aged 8–13 years were recruited to participate in this cross-sectional study from a larger study of Black primary caregivers and children from a low-income, urban population with high trauma exposure (Jovanovic et al., 2011; Kamkwalala et al., 2012). Participants provided data in a single study interview. In this sample, 77.5% of the caregivers reported average monthly household income <$2,000, 54.4% reported having either high school or less formal education, 62.4% reported being unemployed, 92% reported experiencing at least one DSM-5 Criterion A trauma, and 82.4% reported being single, divorced, separated or widowed. Chi-square tests indicated that caregivers of girls vs. boys did not significantly differ on any of these measures, all ps > 0.194. Participants were recruited from the waiting rooms of the Primary Care or Obstetrics Gynecology clinics at the Grady Health System and from the Children's Hospital of Atlanta in Atlanta, GA, USA. Exclusion criteria for both caregivers and children included autism spectrum disorders, bipolar or psychotic disorders, and cognitive disability. Prior to participation, all caregivers signed informed consent as well as parental permission for their children, and the children provided study assent approved by the Emory University Institutional Review Board and the Grady Research Oversight Committee.

Assessment of Pubertal Acceleration

Participants' pubertal status was assessed *via* the Pubertal Development Scale (PDS; Petersen et al., 1988). The five-item scale has female and male versions. Prior work has reported that the PDS has good median internal consistency, α = 0.77, with N = 253 (Petersen et al., 1988). In spite of our comparatively

smaller sample, $N = 133$, we observed similar good internal consistency for girls, $\alpha = 0.74$, but not boys, $\alpha = 0.49$. We utilized a coding system to convert the PDS items to a five-point scale that parallels the Tanner stages, which range from one (no development) to five (adult development; Tanner, 1962; Shirtcliff et al., 2009). We then created a pubertal timing score by regressing participants' PDS scores on age and retained the residuals; the residuals were obtained for girls and boys separately (i.e., using sex-stratified models) to account for sex differences in the timing of puberty. Positive residuals indicate more accelerated pubertal timing relative to age (i.e., earlier pubertal timing) and negative residuals indicate less accelerated pubertal timing relative to age (i.e., later timing).

Clinical Assessment

Trauma Exposure

Trained study staff evaluated children's trauma exposure with the child-report Trauma Exposure Screening Inventory (TESI; Ghosh-Ippen et al., 2002), a developmentally-appropriate 18-item scale that has good psychometric properties (Ribbe, 1996). Staff only recorded exposure for traumas that met the definition of Criterion A trauma per their clinical judgment and experience working with children from this population (American Psychiatric Association, 2013), with the exception of two TESI items are not Criterion A traumas ("Had someone in your family ever been put in jail or prison?" and "Seen or heard people attacking each other for real on television or the internet?"). Total trauma exposure was calculated as the number of types of traumas that the child endorsed.

Anxiety Symptoms

Children's anxiety symptoms were assessed using the Child Rating Scales from the Behavioral Assessment System for Children, Second Edition (BASC-2; Reynolds and Kamphaus, 2004). Age- and sex-normed T scores were generated using automated scoring software and ranged from 34 to 82. Scores between 60 and 69 were considered at-risk and scores above 70 were considered clinically significant; 13.4% ($n = 16$) of children were in the at-risk category and 4.1% ($n = 5$) qualified as clinically significant.

Statistical Analysis

All analysis were conducted using SPSS version 25 and PROCESS for SPSS version 2.16.3. Results were considered significant only if the 95% confidence intervals did not contain 0 or if alpha < 0.05 for p-values. Confidence intervals for the moderation and statistical mediation models were generated from 5,000 bootstrap resamples. Sex differences in the association between: (a) trauma exposure and pubertal timing; and (b) pubertal timing and anxiety symptoms, were tested with moderation models that included sex as the moderator. Significant moderations by sex were followed by sex-stratified hierarchical multiple regression analyses to test whether household income, trauma exposure, and accelerated pubertal timing predicted anxiety symptoms. A statistical mediation was conducted to evaluated the hypothesis that pubertal timing would partially mediate the association between trauma and anxiety symptoms for girls.

RESULTS

Sex Similarities and Differences in the Sample

Girls and boys did not significantly differ in age, trauma exposure, or anxiety symptoms, all ps > 0.417. Body mass index (BMI) was higher in girls than boys, but this difference was not significant, $p = 0.072$. Girls had significantly more advanced pubertal status and accelerated pubertal timing relative to boys, both ps < 0.038, indicating that girls' pubertal development was generally more advanced than boys'. See **Table 1** for descriptive statistics and results of independent samples t-tests, and **Figure 1** for histograms of pubertal status, anxiety symptoms, and trauma exposures. Results of a chi-square test of independence indicated that caregivers of girls and boys reported similar household incomes, $\chi^2_{(4,N=124)} = 1.08$, $p = 0.897$. **Table 2** reports Pearson correlations between all variables of interest for girls (Panel A) and boys (Panel B) separately. Trauma exposure was positively associated with girls' pubertal development, $r_{(62)} = 0.33$, $p = 0.008$, and pubertal timing, $r_{(62)} = 0.29$, $p = 0.023$. In contrast, trauma exposure was not associated with boys' pubertal development, $r_{(62)} = -0.16$, $p = 0.207$, but was negatively associated with pubertal timing, $r_{(62)} = -0.36$, $p = 0.004$.

Association Between Trauma and Pubertal Timing Is Moderated by Sex

We tested for sex differences in the relationship between child-reported trauma exposure and accelerated pubertal timing using

TABLE 1 | Descriptive statistics for girls ($n = 64$) and boys ($n = 67$) and results of independent samples t-tests.

	Boys		Girls		
	Mean	SD	Mean	SD	t-tests t(df)
Age (months)	122.15	18.53	119.63	16.97	0.81 (129)
Body mass index	19.40	4.41	21.20	6.34	−1.82 (120)$^+$
Trauma exposure	4.23	2.96	4.41	2.64	−0.37 (123)
Anxiety	49.40	11.08	48.73	9.71	0.35 (119)
Pubertal development	1.99	0.95	2.35	1.01	−2.10 (128)*
Pubertal timing	−0.20	0.87	0.22	0.81	−2.84 (128)**

*Note: $^+$indicates $p < 0.10$, *indicates $p < 0.05$, **indicates $p < 0.01$.*

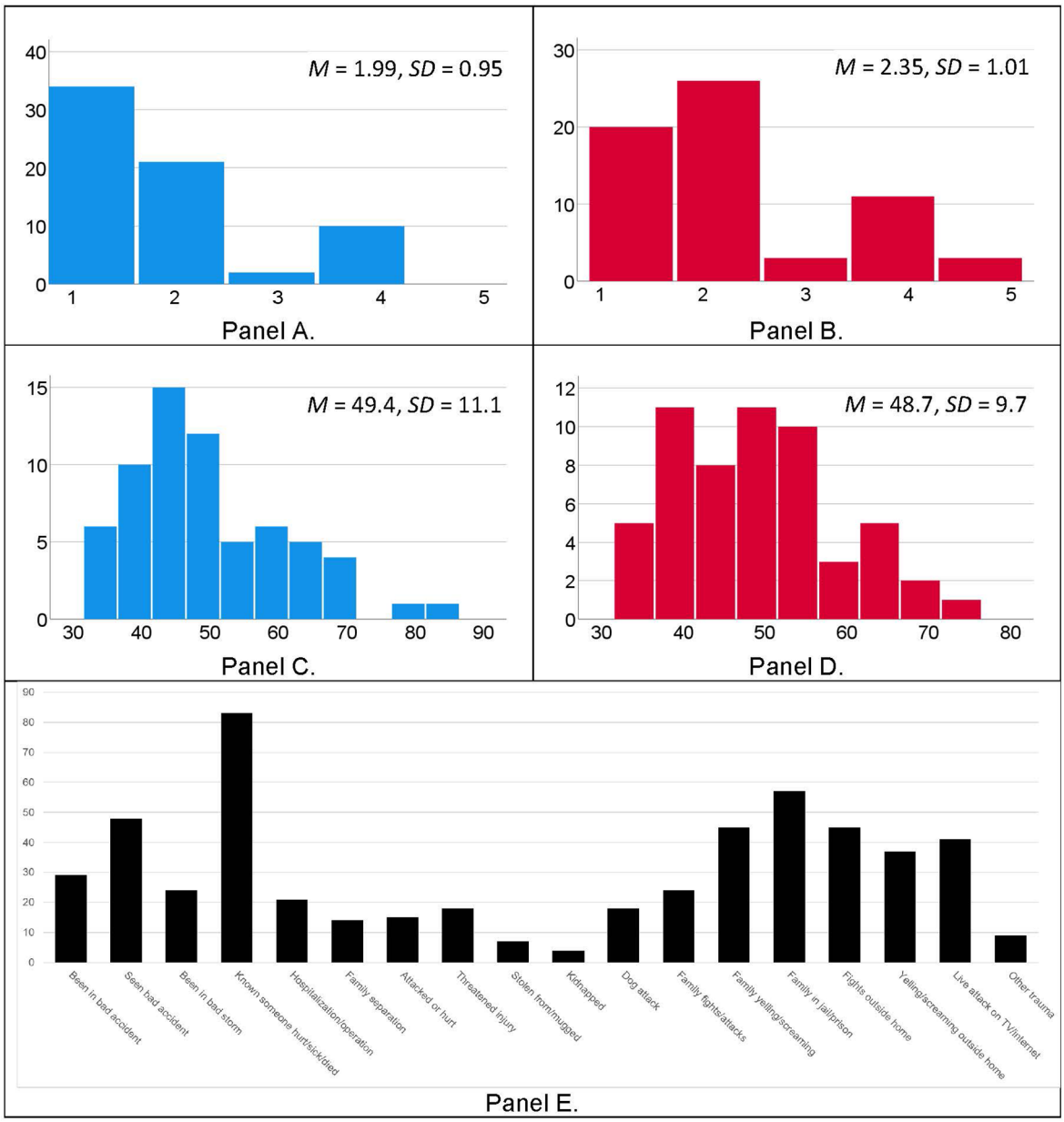

FIGURE 1 | Histograms depicting pubertal development scores for boys (Panel **A**, blue bars) and girls (Panel **B**, red bars), and age- and sex-normed anxiety *T*-scores from the BASC-2 for boys (Panel **C**, blue bars) and girls (Panel **D**, red bars). Histogram of child-reported exposure to traumatic events, per the Traumatic Exposure Screening Inventory (TESI; Panel **E**, black bars).

a moderation model (PROCESS Model 1; see **Figure 2**, Panel A). The model was significant overall, $F_{(3,120)} = 8.69$, $R^2 = 0.18$, $p < 0.001$, and there was a significant interaction between trauma exposure and sex, $t = 3.71$, $p < 0.001$, 95% confidence interval (CI) [0.09, 0.30]. The conditional effect of trauma exposure was associated with pubertal timing for girls, $b = 0.09$, $SE = 0.04$, $p = 0.022$, 95% CI [0.01, 0.17], whereas for boys there was an inverse relationship between trauma exposure and pubertal timing, $b = -0.10$, $SE = 0.04$, $p = 0.003$, 95% CI [−0.17, −0.04]. Because BMI differed between girls and boys, we also conducted this moderation analysis with BMI included as a covariate. The model was significant, $F_{(4,114)} = 8.39$, $R^2 = 0.23$, $p < 0.001$, and the interaction between trauma and sex remained significant, $t = 3.64$, $p < 0.001$, 95% CI [0.08, 0.29], but BMI was not a significant predictor, $t = 1.88$, $p = 0.063$, 95% CI [−0.001, 0.05]. These results suggest that trauma exposure is associated with accelerated pubertal timing in girls and with delayed pubertal timing in boys within this cohort, as shown in **Figure 3**, Panel A, and that this sex difference remained when controlling for BMI.

TABLE 2 | Correlations between all study variables for girls (Panel A; $n = 64$) and boys (Panel B; $n = 67$).

	1. Age	2. Income	3. Trauma	4. Pubertal development	5. Pubertal acceleration	6. Anxiety symptoms
Panel A						
1.	–	0.047	0.249*	0.612**	0.191	−0.263
2.	–	–	−0.131	0.190	0.201	−0.087
3.	–	–	–	0.334**	0.289*	0.369**
4.	–	–	–	–	0.893**	0.156
5.	–	–	–	–	–	0.343*
Panel B						
1.	–	0.082	0.321*	0.420**	−0.125	0.004
2.	–	–	−0.158	0.253*	0.230	0.113
3.	–	–	–	−0.163	−0.358**	0.242
4.	–	–	–	–	0.848**	−0.059
5.	–	–	–	–	–	−0.070

*Note: *indicates $p < 0.05$, **indicates $p < 0.01$.*

Association Between Pubertal Timing and Anxiety Symptoms Is Moderated by Sex

We also tested for sex differences in the relationship between pubertal timing and anxiety symptoms using a moderation model (PROCESS Model 1; see **Figure 2**, Panel B). The model was not significant overall, $F_{(3,117)} = 2.08$, $R^2 = 0.05$, $p = 0.106$, however, the interaction between trauma exposure and sex was significant, $t = 2.18$, $p = 0.031$, 95% confidence interval (CI) [0.48, 9.71]. For girls, there is a significant positive association between pubertal timing and anxiety symptoms, $b = 4.15$, $t = 2.40$, $p = 0.018$, 95% CI [0.72, 7.58]. This association was not significant for boys, $b = -0.94$, $t = -0.60$, $p = 0.547$, 95% CI [−4.04, 2.15]. See **Figure 3**, Panel B.

Regression Models Predicting Anxiety Symptoms in Girls and Boys

Sex moderated the trauma-pubertal timing and the pubertal timing-anxiety associations, therefore, we stratified by sex for subsequent analyses of the associations between trauma exposure, pubertal timing, and self-reported anxiety symptoms. Hierarchical regressions of anxiety symptoms as the outcome included the following predictors: household income in block 1, trauma exposure in block 2, and pubertal timing in block 3. For boys, the overall model was not significant, $F_{(3,57)} = 1.43$, $R^2 = 0.07$, $p = 0.245$. Household income was not significant, $\beta = 0.09$, $p = 0.487$. Trauma exposure approached statistical significance, with greater exposure predicting more anxiety symptoms, $\beta = 0.24$, $p = 0.087$. Pubertal acceleration was not significant, $\beta = -0.04$, $p = 0.761$. For girls, this regression model was significant, $F_{(3,52)} = 5.23$, $R^2 = 0.24$, $p = 0.003$. Household income did not predict anxiety symptoms, $\beta = -0.09$, $p = 0.536$. Trauma was associated with higher anxiety, $\beta = 0.35$, $p = 0.010$, as was pubertal acceleration, $\beta = 0.36$, $p = 0.009$. See **Table 3** for model details.

Results of the hierarchical regression for girls indicated that when pubertal acceleration was added to the model in block 3 the association between trauma exposure and anxiety symptoms was reduced, from $\beta = 0.35$ to $\beta = 0.27$. To further delineate these associations, we conducted a statistical mediation analysis with trauma exposure as the predictor, pubertal acceleration as the mediator, and anxiety as the dependent variable (**Figure 2**, Panel C). This model was significant, $F_{(2,53)} = 7.01$, $p = 0.002$, $R^2 = 0.21$. The direct effect of trauma remained significant, $\beta = 1.15$, SE = 0.47, 95% CI [0.22, 2.09]. Pubertal timing partially statistically mediated the association between trauma exposure and anxiety symptoms, $\beta = 0.219$, SE = 0.152, $p = 0.023$, 95% CI [0.22, 2.09].

DISCUSSION

Most prior studies examined the causes and consequences of accelerated pubertal timing separately (Henrichs et al., 2014; Negriff et al., 2015; Noll et al., 2017; Ullsperger and Nikolas, 2017; Suglia et al., 2020). One result of this approach is ambiguity about whether ELS-linked acceleration of pubertal timing is independent of or an indirect pathway through which ELS impacts mental health. The present cross-sectional study examined trauma exposure, pubertal timing, and internalizing symptoms simultaneously in Black girls and boys in order to test: (a) whether trauma exposure is associated with anxiety symptoms indirectly through accelerated pubertal timing; and (b) if these associations differ by sex. To our knowledge, this is one of the first studies to directly test all three of these paths (from trauma to anxiety, trauma to accelerated pubertal timing, and accelerated pubertal timing to anxiety) within a sample of Black girls and boys.

The results of our moderation analyses provide evidence for sex-specific associations between trauma and accelerated pubertal timing. Interestingly, these results suggest that trauma exposure may be associated with accelerated pubertal timing in girls but not boys in this sample. This result is consistent with multiple prior studies that indicate accelerated pubertal timing following threat-related ELS for girls (Negriff et al., 2015; Noll et al., 2017; Copeland et al., 2019; Gur et al., 2019; Sumner et al., 2019). Interestingly, for boys greater trauma exposure was associated with later pubertal onset relative to boys with less trauma exposure in this cohort. While some studies report that ELS is linked to accelerated pubertal timing in boys (Gur et al., 2019; Sumner et al., 2019), other studies have indicated that for

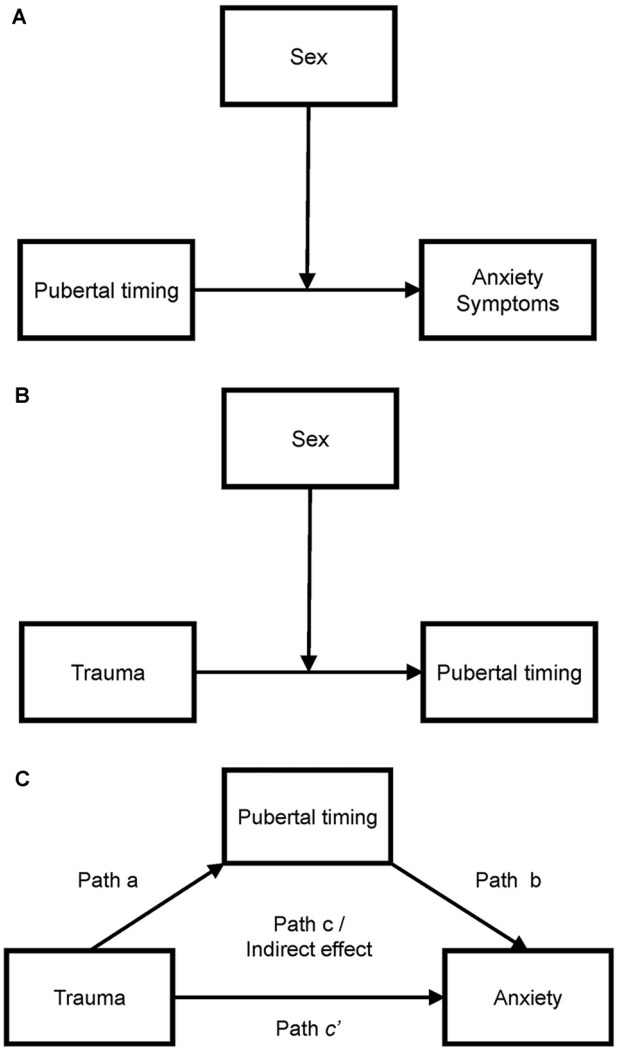

FIGURE 2 | Moderation model used to test for sex differences in the association between trauma exposure and pubertal timing (Panel **A**), the association between pubertal timing and anxiety symptoms (Panel **B**), and statistical mediation model for the associations between trauma, pubertal timing, and anxiety symptoms in girls (Panel **C**).

boys ELS may be more often linked to faster progression through puberty once it begins rather than early onset (Negriff et al., 2015). It is possible that if we had continued to follow these boys into their teenage years those with more trauma exposure would display faster pubertal tempo relative to those with low or no trauma. However, our results parallel those from a growing number of studies that report slower pubertal timing in boys who experience more ELS relative to those who experience less (Semiz et al., 2009; Johnson et al., 2018; Suglia et al., 2020).

Interpretation of our results is complicated by the significant sex differences in pubertal status. Specifically, the boys were predominantly in early puberty, making it difficult to determine if our results reflect that: (a) trauma exposure is associated with delayed pubertal onset in Black boys; or (b) we did not capture a wide enough range of puberty in boys to accurately detect the association between trauma and puberty. The PDS internal consistency measures were good for girls but less so for boys. This suggests that our measurement of puberty may have been less reliable for boys, perhaps in part because of their relatively early pubertal development. However, prior studies have also reported that ELS is linked to earlier pubertal timing in girls but delayed timing in boys from three different populations (Semiz et al., 2009; Johnson et al., 2018; Suglia et al., 2020). Studies that examine the associations between trauma, pubertal timing, and mental health in Black girls and boys at equivalent stages of development are needed to determine if the present results reflect true sex differences in the association between trauma and puberty or are an artifact of testing girls and boys at different stages of puberty.

Puberty is studied much less in Black boys relative to other groups. Data suggest that, like Black girls, their pubertal timing tends to be earlier than other racial and ethnic groups (Sun et al., 2002). Some studies that include Black boys report that threat/trauma exposure is associated with earlier pubertal timing, although they have not reported these results by race/ethnicity (Gur et al., 2019; Sumner et al., 2019). One of the only large studies we are aware of that specifically examined pubertal timing in Black boys (N = 375) found that two environmental factors (harsh community environment and harsh/inconsistent parenting) interacted with negative emotionality, such that the environmental risk factors only predicted early pubertal timing at age 13 for boys with high negative emotionality (Kogan et al., 2015). These intriguing results suggest that some individual difference factors, such as negative or positive emotionality, may buffer Black boys against the effects of trauma exposure on pubertal timing.

The sex-stratified regression models indicated that pubertal timing explained significant variance in anxiety symptoms for girls but not boys, even after household income and trauma exposure were included as predictors in the models. We also found that this association between pubertal timing and anxiety symptoms partially statistically mediated the significant association between trauma and anxiety for girls. Two relevant sex differences may contribute to these results. First, one source of risk related to early pubertal timing is a mismatch between the capabilities of the child (e.g., to regulate emotions) and the effects of hormonal increases and fluctuations that characterize puberty (Petersen and Taylor, 1980; Brooks-Gunn et al., 1985; Angold et al., 1999). Later puberty in boys vs. girls may buffer boys from some mental health risks because their cognitive development is more advanced prior to the onset of puberty. Second, puberty produces different hormonal milieus for girls and boys, and evidence suggests that fluctuations in estradiol levels in females confers risk for anxiety disorders (Angold et al., 1999; Toufexis et al., 2006; Maeng and Milad, 2015). Third, the early onset of puberty is also associated with a mismatch between the child's social knowledge and skills and how the child is perceived and treated in social contexts. As with cognitive development, later puberty may be protective because more social abilities are acquired prior to puberty. For girls, the physical changes associated with puberty, such as breast development, can translate to sexual attention and related

TABLE 3 | Hierarchical regression results for boys (Panel A) and girls (Panel B) for the dependent variable anxiety symptoms.

	Model 1			Model 2			Model 3		
Variable	**B**	***SE B***	***β***	**B**	***SE B***	***β***	**B**	***SE B***	***β***
Panel A									
Income	0.89	1.24	0.10	1.24	1.23	0.13	1.33	1.27	0.14
Trauma				0.99	0.51	0.25^{+}	0.94	0.54	0.24^{+}
Pubertal acceleration							−0.61	1.99	−0.04
R^2		−0.01			0.04			0.02	
R^2 Change		-			0.06			0.00	
F for change in R^2		0.51			3.72^{+}			0.09	
Panel B									
Income	−0.71	1.13	−0.09	−0.36	1.08	−0.04	−1.24	1.07	−0.15
Trauma				1.34	0.05	0.35*	1.01	0.49	0.27*
Pubertal acceleration							4.37	1.62	0.36**
R^2		−0.012			0.01			0.20	
R^2 Change		-			0.12			0.11	
F for change in R^2		0.39			7.08*			7.30**	

*Note: $^{+}$indicates $p < 0.10$, *indicates $p < 0.05$, **indicates $p < 0.01$.*

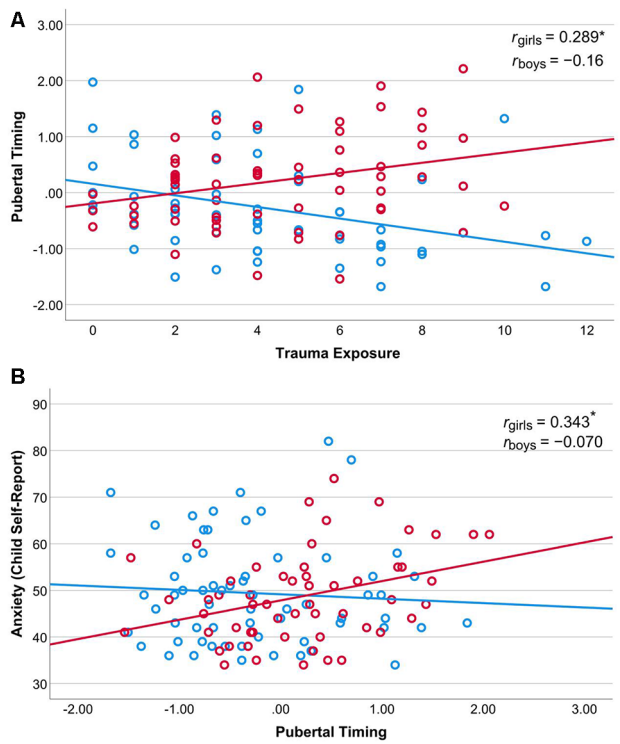

FIGURE 3 | Association between trauma exposure (*x*-axis) and pubertal timing (y-axis) for girls and boys (Panel **A**). Association between pubertal timing (*x*-axis) and anxiety symptoms (*y*-axis) for girls and boys (Panel **B**). *Note*: *indicates $p < 0.05$.

body image issues that may contribute to anxiety symptoms, particularly when cognitive and social development is less advanced (Lindberg et al., 2007; Petersen and Hyde, 2009; Skoog et al., 2016). The majority of studies that examine associations between early adversity, puberty timing, and psychopathology include only girls; much less is known about this phenomenon in boys (Ullsperger and Nikolas, 2017; Joos et al., 2018). Additional research will be needed to determine what causes and consequences of accelerated pubertal timing are sex-general or sex-specific.

We were not directly able to address mechanisms through which trauma impacts pubertal timing, however, putative mechanisms include chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis by traumatic stressors. Stressors, including traumatic events, activate the HPA axis, which controls the production of glucocorticoids and thereby impacts activity in both the peripheral and central nervous systems (Ulrich-Lai and Herman, 2009). The SAM axis reacts to threats in the environment by activating the sympathetic nervous system and initiating a rapid "fight or flight" response (Ulrich-Lai and Herman, 2009). Both the HPA axis and the SAM innervate the hypothalamus, a brain structure that controls the initiation of puberty. Extreme and repeated stress, such as that experienced during traumatic episodes, could modulate hypothalamic structure and function and thereby impact hypothalamic control of pubertal timing.

The interplay between the HPA and HPG axes is a potential route through which ELS could impact pubertal timing (Marceau et al., 2014, 2015; Negriff et al., 2015). There is evidence for inverse coupling of the HPA and HPG axes in adulthood, such that stress hormones suppress gonadal hormones (Stratakis and Chrousos, 1995; Romeo, 2005; Terburg et al., 2009). However, some evidence suggests that, unlike in adulthood, there is a positive coupling between the HPA and HPG axes during adolescence (Marceau et al., 2014), which may be more robust in girls than boys (Marceau et al., 2015). Some evidence indicates that ELS exposure modulates the cortisol awakening response according to pubertal status (King et al., 2017). A study of children in the same age range (8–13 years) as the present study reported that for boys, but not girls, community violence exposure in the past 12 months was negatively correlated with cortisol reactivity in the context of completing the Trier Social Stress Test (Peckins et al., 2012). This finding raises the possibility that violence and trauma exposure during middle childhood and early adolescence impact the hormonal milieu differently for girls vs. boys, which in turn drives sexually

dimorphic effects on pubertal timing, as the results of our study suggest.

Studies have consistently indicated that Black girls start and complete puberty earlier than other racial and ethnic groups (Freedman et al., 2002; Wu et al., 2002; Chumlea et al., 2003; Anderson and Must, 2005; Rosenfield et al., 2009). Our findings suggest that well-documented racial disparities in ELS and trauma (Merrick et al., 2019) may contribute to earlier average pubertal timing in Black girls relative to other groups. More specifically, the fact that Black girls typically experience more ELS and trauma than peers from other groups may contribute to the observed racial differences in pubertal timing. This possibility should be explored further, particularly given that early pubertal timing is linked to increased risk for poor mental (e.g., Ullsperger and Nikolas, 2017) and physical health outcomes (Elks et al., 2013; Prentice and Viner, 2013; Day et al., 2015).

Our results add to the existing literature on pubertal timing by focusing on the impact of trauma exposure within a sample of Black children from low-SES families that experience high levels of adversity. This design avoids potential confounds, such as collinearity between trauma exposure and SES, that complicate the interpretation of the results. However, there are several limitations of the present study that should be considered. First, we rely on a self-report measure of pubertal status, rather than employing multiple measures, such as gonadal hormone levels and physical examinations by trained health care providers. Although data indicate that self-report is a reliable way to measure pubertal status (Shirtcliff et al., 2009), future studies will be better able to probe the mechanisms underpinning accelerated pubertal timing if they employ multiple measures of pubertal status. For instance, assessment of dehydroepiandrosterone, estradiol, and testosterone levels in addition to self-report measures could provide insight into the relationships between ELS, adrenarche, and gonadarche. Second, the age range of our sample (8- to 13-years-old) does not capture the full window of puberty, particularly for boys who generally start puberty later than girls (Dorn and Biro, 2011). Boys in this sample were earlier in puberty than the girls; this complicates the interpretation of the sex differences we report. Ideally, future studies will follow children from ∼7 years, when adrenarche begins, through the teenage years. Third, the cross-sectional study design does not allow us to directly examine causation or changes in pubertal status within individuals. This limits our ability to address causation or the directionality of the reported associations, particularly for results of the statistical mediation analysis. These limitations highlight the need for longitudinal, prospective studies that examine adversity, trauma exposure, and pubertal timing.

The results of the current study add to growing evidence that psychosocial factors can alter developmental timing in a manner that increases the risk for poor mental health outcomes. In the context of recent work that has identified associations between threat-related adversity and accelerated pubertal development, our results provide evidence that traumas that involve threat may have sex-specific effects on pubertal timing in Black children. Our results are also consistent with the hypothesis that trauma increases the risk for psychopathology in girls both directly and indirectly *via* acceleration of pubertal development. These results highlight the need to identify the mechanisms through which ELS and trauma modulate pubertal timing. Identification of these mechanisms may help to resolve the somewhat mixed findings regarding factors linked to pubertal acceleration in girls and boys. A more complete understanding of how ELS and trauma are transduced into accelerated development is both a key task for developmental science and a necessary step towards identifying targets for intervention in children who experience ELS and trauma.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University Institutional Review Board Grady Research Oversight Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AS: conceptualization, data curation, formal analysis, roles/writing—original draft, and visualization. VM: conceptualization, writing—review and editing. JS: investigation, writing—review and editing. AP: data curation, investigation, writing—review and editing. TJ: conceptualization, funding acquisition, writing—review and editing. All authors contributed to the article and approved the submitted version.

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Germ Cell Drivers: Transmission of Preconception Stress Across Generations

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Exposure to stress can accelerate maturation and hasten reproduction. Although potentially adaptive, the trade-off is higher risk for morbidity and mortality. In humans, the intergenerational effects of stress have been demonstrated, but the precise mechanisms are unknown. Strikingly, even if parental stress occurs prior to conception, as adults, their offspring show worse mental and physical health. Emerging evidence primarily from preclinical models suggests that epigenetic programming may encode preconception stress exposures in germ cells, potentially impacting the phenotype of the offspring. In this narrative review, we evaluate the strength of the evidence for this mechanism across animals and humans in both males and females. The strongest evidence comes from studies of male mice, in which paternal preconception stress is associated with a host of phenotypic changes in the offspring and stress-induced changes in the small non-coding RNA content in sperm have been implicated. Two recent studies in men provide evidence that some small non-coding RNAs in sperm are responsive to past and current stress, including some of the same ones identified in mice. Although preliminary evidence suggests that findings from mice may map onto men, the next steps will be (1) considering whether stress type, severity, duration, and developmental timing affect germ cell epigenetic markers, (2) determining whether germ cell epigenetic markers contribute to disease risk in the offspring of stress-exposed parents, and (3) overcoming methodological challenges in order to extend this research to females.

Keywords: stress, trauma, epigenetics, small non-coding RNA, germline, sperm, extracellular vesicles, oocytes

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INTRODUCTION

Exposure to chronic stress or trauma, particularly in early life, prompts neural, developmental, physiological, and behavioral changes (Maccari et al., 2014; Cameron and Schoenfeld, 2018; Ellis and Del Giudice, 2019). These changes may be adaptive if they increase the likelihood of surviving long enough to reproduce (e.g., by increasing vigilance to threat) and/or accelerate development in order to reproduce sooner (e.g., by reaching puberty earlier). Evidence in humans shows that

early life stress does indeed accelerate development, particularly when the adversity is threat-related (e.g., violence) rather than deprivation-related (e.g., food insecurity) (Belsky, 2019; Sumner et al., 2019). In humans, early life adversity is associated with earlier age of reproductive events – puberty, menarche, parturition, and menopause – but also accelerated aging at the cellular level (Wilson and Daly, 1997; Lawlor et al., 2003; Jorm et al., 2004; Bogaert, 2005; Chisholm et al., 2005; Hardy and Kuh, 2005; Mishra et al., 2007; Pesonen et al., 2008; Nettle, 2010; Belsky et al., 2017; Lei et al., 2018; Magnus et al., 2018; Ridout et al., 2018). Early life adversity and its developmental consequences (i.e., accelerated development and aging) are associated with a wide range of poor mental and physical health outcomes and earlier mortality (Felitti et al., 1998; Golub et al., 2008; Thomas et al., 2008; Jones et al., 2009; Wegman and Stetler, 2009; Kessler et al., 2010; McLaughlin et al., 2010, 2016; Shuster et al., 2010; Ma et al., 2011; Rich-Edwards et al., 2012; Widom et al., 2012; Charalampopoulos et al., 2014; Haycock et al., 2014; Janghorbani et al., 2014; Bellis et al., 2015; Duncan et al., 2015; Rode et al., 2015; Campbell et al., 2016; Chen E. et al., 2016; Lei et al., 2018; Wolkowitz, 2018; Carter et al., 2019). Accelerated development may enhance reproductive fitness in a stressful environment but may result in critical systems developing too quickly (e.g., synaptic pruning of the brain), leading to less-than-optimal development. These costly tradeoffs are best understood in an evolutionary context. Life history theory explains why harsh, unstable, and unpredictable conditions tend to produce a faster life strategy – a "live fast, die young" approach, characterized by accelerated maturation, hastened reproduction, and a shortened lifespan.

Programming of a fast life strategy may begin preconception. In humans, parental preconception trauma is associated with alterations in the hypothalamic-pituitary-adrenal (HPA) stress axis in their children (Yehuda et al., 2007a,b; Bader et al., 2014). This may lead to blunted cortisol levels and enhanced negative feedback, which could prepare offspring for life under stressful conditions. However, these stress-induced adaptations may come at a cost for offspring too. Across a wide range of parental stress exposures – genocide, war, combat, famine, and childhood maltreatment – preconception stress is associated with increased risk for negative mental and physical health outcomes in offspring despite not being directly exposed to the stressor (Yehuda et al., 1998; Dubowitz et al., 2001; Kaati et al., 2002; Pembrey et al., 2006; Dekel and Goldblatt, 2008; Miranda et al., 2011; Field et al., 2013; Veenendaal et al., 2013; Bezo and Maggi, 2015). The observation that parental preconception stress negatively affects offspring may be explained by various social and environmental factors, such as prenatal programming (e.g., exposures *in utero*), disadvantaged environments, poorer parenting behavior, family dysfunction, insecure attachment styles in the parent(s), and parental mental health problems (Dubowitz et al., 2001; Dekel and Goldblatt, 2008; Sharkey, 2008; Miranda et al., 2011; Field et al., 2013; Moog et al., 2016; Cooke et al., 2019). However, emerging evidence in animal studies supports a biological pathway by which parents may pass their preconception stress experiences to their offspring through epigenetic germ cell transmission (e.g., Rodgers et al., 2013, 2015; Dias and Ressler, 2014; Gapp et al., 2014; Chan et al., 2020). The strongest evidence for this comes from studies in male mice. These studies have shown that paternal stress experiences are linked with epigenetic changes associated with sperm and subsequent phenotypic alterations in the offspring (e.g., Rodgers et al., 2013, 2015; Gapp et al., 2014; Chan et al., 2020).

Epigenetic changes modify the expression of a gene without altering its underlying genetic sequence (Jaenisch and Bird, 2003). Because environmental exposures induce epigenetic changes, they allow the organism to flexibly and rapidly adapt to the environment, particularly harsh environments. Several types of key epigenetic changes can be altered by the environment, including DNA methylation, histone modifications, and non-coding RNA (ncRNA).

The evidence that stress can modify germ cell epigenetic markers comes from studies investigating DNA methylation and sncRNA. Although paternal genome DNA methylation marks undergo near complete erasure and reprogramming following fertilization, the epigenome is not entirely reset, suggesting that certain epigenetic patterns could be transmitted to offspring through the effects of sperm DNA methylation (Hackett et al., 2013; Ly et al., 2015). However, exactly which DNA methylation patterns escape erasure or are reprogrammed remains to be elucidated. Nevertheless, it is also plausible that DNA methylation changes in the paternal germline could also be translated into a different type of "signal" at conception that is then perpetuated in embryo development, ultimately resulting in a phenotypically meaningful biological change. However, the mechanisms by which specific methylation changes could be propagated are not known. Until the mechanisms are understood, caution should remain as to whether changes to specific DNA methylation marks detected in sperm can be transmitted to offspring.

Unlike DNA methylation, sncRNA are not required to be a self-perpetuated signal following fertilization or during development as they have an immediate function at conception and, therefore, have emerged as causal agents of stress signal transmission (Meikar et al., 2011; Morgan et al., 2019, 2020; Chan et al., 2020). In brief, sncRNA are short RNA that are less than 200 nucleotides in length and are considered "non-coding" because they typically are not translated into proteins (Marcho et al., 2020). Nevertheless, sncRNA regulate the expression of genes and serve as epigenetic mediators of environmental exposures (Fullston et al., 2013; Huang et al., 2013; Rodgers et al., 2013; Chen Q. et al., 2016; Cropley et al., 2016; de Castro Barbosa et al., 2016; Sharma et al., 2016; Conine et al., 2018). Two types of sncRNA that we will discuss further are microRNA (miRNA) and transfer RNA (tRNA), which are typically found in sperm as tRNA fragments (Kiani and Rassoulzadegan, 2013; Chen Q. et al., 2016; Cropley et al., 2016; Sharma et al., 2016, 2018; Selvaraju et al., 2018).

Here, we present evidence that stress is associated with changes in germ cell DNA methylation marks and sncRNA content. These epigenetic changes in the germ cell correspond with alterations in offspring phenotype, with the implication that these epigenetic modifications transmit a "stress" signal to impact offspring development, ultimately altering behavioral,

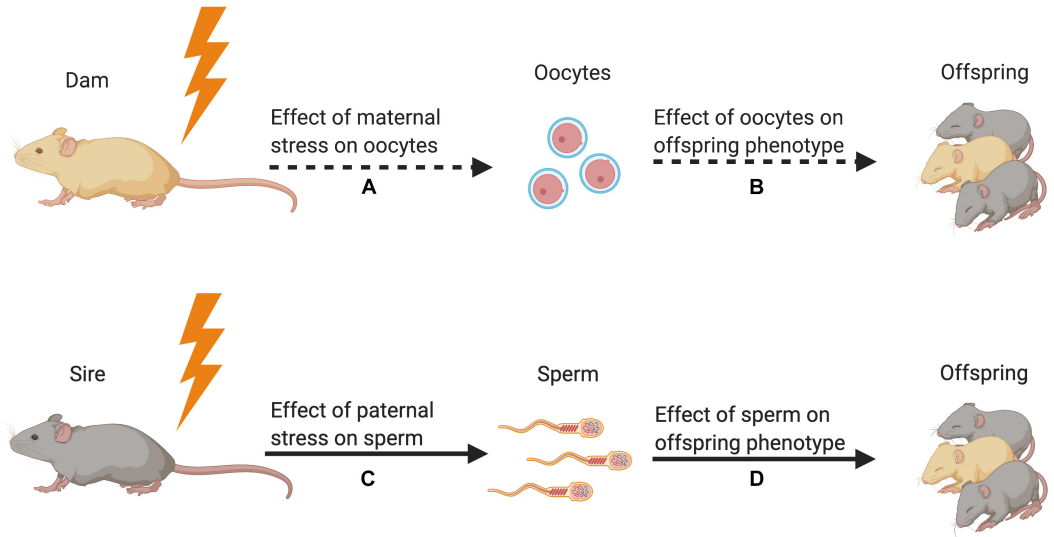

FIGURE 1 | Strength of the evidence for germline transmission of parental stress on offspring phenotype in animal models. **(A)** Only one study has demonstrated an effect of maternal preconception stress on oocytes (Zaidan et al., 2013). This study showed that stress increased corticotropin releasing factor type 1 messenger RNA but did not reveal a change in epigenetic signals. **(B)** No studies have tested for the effect of stress-induced oocyte epigenetic changes on offspring phenotype. **(C)** Many studies have demonstrated that paternal preconception stress affects sperm DNA methylation and small non-coding RNA content (Franklin et al., 2010; Rodgers et al., 2013; Gapp et al., 2014, 2016; Bohacek et al., 2015; Wu et al., 2016; Chan et al., 2020). **(D)** Multiple studies have used *in vitro* fertilization, intracytoplasmic sperm injection, and microRNA microinjection to demonstrate that stress-induced alterations in sperm sncRNA content affect offspring phenotype (Dias and Ressler, 2014; Gapp et al., 2014, 2018; Rodgers et al., 2015; Wu et al., 2016; Chan et al., 2020). Figure created using BioRender.com.

physiological, neural, and/or cognitive outcomes (see **Figure 1**). The scope of our narrative review is limited to studies examining epigenetic and molecular modifications in germ cells, presented separately for oocytes and sperm (see **Table 1** for an overview of the evidence). Currently, the only evidence of epigenetic inheritance is intergenerational, meaning epigenetic transmission is from parent (F0 generation) to offspring (F1), rather than transgenerational, in which epigenetic effects would have to be shown to be propagated to at least the F2 generation. Because the vast majority of studies on preconception stress effects on germ cells have been conducted in males, we focus on sperm studies to highlight factors critical to producing epigenetic changes in sperm and present evidence that these epigenetic changes impact offspring outcomes. Furthermore, because stress-induced changes in sncRNA content in sperm are the epigenetic mechanism most likely to be able to impact offspring (based on our current understanding of which epigenetic markers get transmitted through the germ cell), we narrow in on recent research showing how sperm may acquire these epigenetic changes. Finally, we discuss current knowledge gaps and provocative future research directions.

TRANSMISSION OF MATERNAL PRECONCEPTION STRESS TO OFFSPRING VIA THE GERM CELL

To our knowledge, only one study to date has examined the effect of preconception stress on oocytes (Zaidan et al., 2013). In this study, F0 adolescent female rats were exposed to chronic unpredictable stress for 7 days, and then stress-exposed and control females either had oocytes extracted or were bred (Zaidan et al., 2013). Reverse-transcription PCR was used to measure corticotropin-releasing factor 1 (CRF1) messenger RNA (mRNA) expression in oocytes and in various brain regions in the offspring. Oocyte expression of CRF1 mRNA was 18.5 times greater in the oocytes of stress-exposed females compared to controls. In the brains of adult offspring from stress-exposed and control dams, group differences in CRF1 expression in the amygdala and frontal cortex emerged but were dependent on the extent to which offspring were exposed to stress in adulthood.

While this study is compelling, it does not provide mechanistic evidence that maternal preconception stress programs offspring outcomes through epigenetic changes to the oocyte. The intrauterine environment and maternal behavior are important confounds that make studying maternal transmission of stress experienced before conception a substantial challenge. These confounds can be reduced but require the embryo to be transferred to the uterus of a non-stressed surrogate female and the neonates to be cross fostered by a non-stressed surrogate mother. The study discussed above did not control for either of these factors.

In humans, testing whether oocytes show epigenetic changes in response to stress is nearly impossible. In the US and in other countries, access to oocytes is extremely limited owing to the fact that federal funds cannot be used to provide compensation for the donation of oocytes for research (Spar, 2007; Safier et al., 2018). As a result, no studies have assessed stress-induced epigenetic changes to human oocytes. Given challenges of studying this in

TABLE 1 | Summary of studies testing for stress-induced or stress hormone-induced molecular changes in germ cells.

	Species	Stress paradigm	Developmental stage	Molecular changes
Oocytes				
Zaidan et al., 2013	Mice	Chronic unpredictable stress (1 week)	Adulthood	18.5-fold greater corticotropin-releasing factor type 1 messenger RNA expression (a key component of the stress response)
Sperm				
Morgan et al., 2020	Humans	Longitudinally measured perceived stress (6 months)	Adulthood	Five miRNA (let-7f-5p, miR-181a-5p, miR-4454, miR-6765-3p, and miR-12136) and four tRNA (tRNA-Gly-GCC-3-1, tRNA-Lys-CTT-1-1, tRNA-Lys-CTT-2-1, and tRNA-Lys-CTT-4-1) fluctuate in response to perceived stress
Dickson et al., 2018	Humans	Retrospectively measured adverse childhood experiences (from ages 0–18)	Childhood and adolescence	Lower levels of multiple miRNA in the miR-449 and miR-34 families
	Mice	Chronic social instability stress (7 weeks)	Puberty into adulthood (PN28-77)	Five-fold lower levels of miR-449a and miR-34c
Chan et al., 2020	Humans	Longitudinally measured perceived stress (6 months)	Adulthood	Broad changes in miRNA in males recovering from stress compared to those with minimal variation in stress over the 6-month period (determined using principal component analysis)
	Mice	Chronic variable stress (4 weeks)	Puberty (PN28-56)	Dramatic differences in miR-9-3p and miR-34c-5p 12 weeks after stress cessation but not after 1 week
Gapp et al., 2014	Mice	Unpredictable maternal separation combined with unpredictable maternal stress (2 weeks)	Juvenile (PN1-14)	2-4-fold higher levels of miR-200b-3p, miR-672-5p, miR-466c-5p, miR-375-3p, and miR-375-5p (the miR-375 family has been implicated in stress response and metabolic regulation)
Franklin et al., 2010	Mice	Unpredictable maternal separation combined with unpredictable maternal stress (2 weeks)	Juvenile (PN1-14)	Higher DNA methylation for the methyl CpG-binding protein 2 gene (a transcriptional regulator that binds methylated DNA) and cannabinoid receptor 1 gene (associated with emotional behavior in rodents) but lower for corticotropin-releasing factor receptor 2 gene (involved in the stress response)
Bohacek et al., 2015	Mice	Unpredictable maternal separation combined with unpredictable maternal stress (2 weeks)	Juvenile (PN1-14)	Lower DNA methylation at the protein kinase C promotor gene implicated in synaptic plasticity and memory performance
Rodgers et al., 2013	Mice	Chronic variable stress (6 weeks)	Puberty into adulthood (PN28-70) or adulthood (PN56-98)	2-5-fold increases in nine miRNAs (miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, and miR-698)
Wu et al., 2016	Mice	Daily restraint (2 weeks)	Adulthood (PN58-72)	Two-fold higher DNA methylation of the *Sfmbt* promotor gene involved in gluconeogenesis
		Daily restraint (2 weeks) with daily treatment with glucocorticoid antagonist	Adulthood (PN58-72)	Glucocorticoid antagonist normalized DNA methylation of the *Sfmbt* promotor gene involved in gluconeogenesis
Petropoulos et al., 2014	Mice	Synthetic glucocorticoid administration (5 days)	Adulthood (PN70-75)	20% increase in global non-CpG methylation in sperm 60 days but not 35 days after treatment with synthetic glucocorticoids
Short et al., 2016	Mice	Corticosterone treatment (4 weeks)	Adulthood (PN70-98)	2-4-fold higher levels of three miRNAs (miR-98, miR-144, and miR-190b), which are thought to interact with multiple growth factors
Dias and Ressler, 2014	Mice	Odor fear conditioning to acetophenone (3 days)	Adulthood (2-months old*)	Lower CpG methylation of the *Olfr151* gene that codes for odor receptors activated by acetophenone

**Age in postnatal days not specified.*
For mice, age at which the stress paradigm occurred is given in both days postnatal (PN) as well as the corresponding developmental stage.

humans, mechanistic research will rely on animal studies for the foreseeable future.

TRANSMISSION OF PATERNAL PRECONCEPTION STRESS TO OFFSPRING VIA THE GERM CELL

Studies on paternal preconception stress provide more evidence of epigenetic germ cell transmission than studies on maternal preconception stress. In this section, we first examine evidence across animal models and human studies showing that stress is associated with epigenetic changes in sperm and phenotypic alterations in offspring. Then, we consider how stress hormones and a stress recovery period are necessary to produce epigenetic changes in sperm; we review the mechanistic evidence that sncRNA changes in sperm drive alterations in offspring phenotype, drawing on studies that use methodologies to isolate the effect of sncRNA on offspring outcomes; and, finally, we discuss how extracellular vesicles in the male reproductive tract may affect the sncRNA content of sperm.

Human Studies

In human studies of male preconception stress on sperm epigenetic changes, research suggests that trauma in childhood and perceived stress in adulthood are associated with changes in the sncRNA content of sperm (Dickson et al., 2018; Morgan et al., 2020). In a cross-sectional study, a higher number of adverse childhood experiences (ACEs; e.g., abuse, neglect, family dysfunction in childhood) correlated with lower levels of multiple miRNA in sperm (Dickson et al., 2018). In the first longitudinal study to measure changes in sperm sncRNA populations over time in healthy adult men, each month for 6 months sperm samples were collected and perceived stress was assessed (Morgan et al., 2020). Five miRNA and four tRNA in sperm fluctuated with perceived stress in a dynamic manner, suggesting that they were responsive to stress. This means that these sncRNA might be able to signal stress in the environment, potentially at conception. Two of the sperm sncRNA identified as stress-responsive have been associated with childhood trauma in a recent study examining plasma sncRNA (Van der Auwera et al., 2019). Identifying sncRNA that respond to stress in human sperm is a first step to translating research from animals to humans. While no human studies have prospectively examined whether stress-induced sperm epigenetic changes are associated with offspring outcomes, research in animals suggests that they may be.

Animal Models

Studying paternal transmission of preconception stress experiences in animal models eliminates major complications of studying this in either female animals (e.g., the fetoplacental environment) or humans (e.g., parental behavior). Male rodents, especially inbred mouse strains, are not typically involved in offspring rearing, allowing researchers to isolate the effect of paternal preconception stress on offspring outcomes. Studies in male animals provide the clearest evidence of germ cell epigenetic transmission of stress experience.

One model of early life stress uses maternal separation combined with unpredictable maternal stress (MSUS) from postnatal days 1 to 14. Four studies using this paradigm found that paternal early life stress exposure was associated with depressive-like behavior in F1 female offspring as well as behavioral despair and reduced fear in F1 offspring of both sexes (Franklin et al., 2010; Gapp et al., 2014, 2018; Bohacek et al., 2015). All four studies identified changes in sperm DNA methylation or sncRNA content, including methylation of genes that regulate the stress response and impart vulnerability to depression-like behavior, at least in mice (Franklin et al., 2010; Valverde and Torrens, 2012; Gapp et al., 2014, 2018; Bohacek et al., 2015). Paternal preconception exposure to MSUS was associated with physical health-related outcomes in offspring (Gapp et al., 2014, 2018). Although early life stress-exposed male mice had normal glucose regulation, their offspring had lower basal and stress-stimulated glucose levels (Gapp et al., 2014). Their offspring also showed signs of hypermetabolism as their body weight was lower despite higher caloric intake. Paternal preconception exposure to MSUS was also associated with worse neural and cognitive outcomes in sires and offspring (Bohacek et al., 2015). Both showed impaired synaptic plasticity and long-term memory. Sperm of F0 males and the hippocampus of F1 offspring showed reduced DNA methylation at the *Prkcc* promotor, a gene implicated in synaptic plasticity and memory performance.

In a separate model, adolescent male mice exposed to chronic social instability stress for 7 weeks showed reductions in the same sperm miRNA (miR-449a and miR-34c) as men exposed to ACEs in the study described previously (Dickson et al., 2018). Importantly, miR-449a and miR-34c are among a small set of miRNA that are not present in oocytes but are delivered to them via sperm during fertilization, suggesting that these miRNA may influence subsequent generations by altering early development (Yuan et al., 2016).

One study tested whether chronic variable stress experienced for 6 weeks, either during puberty or in adulthood, differentially affected F0 sperm miRNA content and F1 offspring stress reactivity (Rodgers et al., 2013). Regardless of when F0 males were exposed to stress, F0 sperm exhibited elevated levels of miRNA, nine that were robustly increased, and F1 offspring showed a significantly blunted HPA stress response to an acute stressor as well as gene set enrichment in stress regulatory brain regions, including the paraventricular nucleus and bed nucleus of stria terminalis (Rodgers et al., 2013; Chan et al., 2020).

In another stress model, adult male mice subjected to daily restraint stress for 2 weeks were bred and then physical health-related outcomes were measured in the offspring (Wu et al., 2016). F0 stress-exposed males exhibited a reduced body weight gain, elevated blood glucose levels, and a two-fold increase in sperm DNA methylation of the *Sfmbt2* promotor compared to control males (Wu et al., 2016). F1 offspring showed a similar increase in blood glucose, an effect that was more pronounced in male than female offspring. The increase in blood glucose may be explained by the fact that follow-up experiments in males showed that they had elevated expression of phosphoenolpyruvate carboxykinase, an enzyme involved in

gluconeogenesis, a process by which glucose is generated from fats and proteins instead of carbohydrates.

Factors Critical to Producing Molecular Changes in Sperm

Stress Hormones

One key assumption in this literature is that heightened exposure to glucocorticoids, both in terms of overall levels and length of exposure, during stress drives lasting epigenetic changes associated with germ cells. Several studies have isolated the specific role of glucocorticoids in this process by either administering synthetic glucocorticoids or utilizing corticosterone treatment. Overall, these studies found that synthetic glucocorticoids and corticosterone treatment were associated with epigenetic alterations in sperm and phenotypic changes in the offspring that mirrored those of paternal stress models (Petropoulos et al., 2014; Short et al., 2016; Wu et al., 2016; Chan et al., 2020). One study further showed that glucocorticoid antagonists given to males during a 14-day period of stress exposure blocked hypermethylation patterns in sperm as well as transmission of the stress-associated phenotypic changes in their offspring, supporting a role of the glucocorticoid receptor in this programming (Wu et al., 2016).

Stress Recovery Period

Exposure to stress or stress hormones are similar in that they do not immediately impart epigenetic changes on sperm. Two studies showed that changes in sperm miRNA content and DNA methylation marks are only detected after a period of recovery from stress or after ceasing synthetic glucocorticoids (Petropoulos et al., 2014; Chan et al., 2020). For example, in one study, sperm miRNA expression showed only minor differences 1 week after chronic stress ended but dramatic differences 12 weeks after stress cessation, and this coincided with the timeline required for transmission of the blunted HPA stress axis phenotype to the F1 offspring (Chan et al., 2020). Overall, a stress recovery period seems to be necessary to alter sperm epigenetic markers and the offspring phenotype. The stress recovery period may serve as a cellular establishment of a new allostatic set point necessary for transcriptional responses, changes in secreted signals, and altered epigenetic marks in germ cells.

Evidence That Molecular Changes in Sperm Matter

Epigenetic alterations associated with sperm are assumed to be causal in the changes detected in the F1 offspring phenotype, but only some studies have actually isolated the role of stress-induced changes in sperm in producing offspring phenotypic changes. *In vitro* fertilization and intracytoplasmic sperm injection methods have been used to eliminate potential confounds – namely, the possibility that mating with a stressed male affects the stress physiology and maternal behavior of the female, which could be driving the observed effects (Dias and Ressler, 2014; Wu et al., 2016; Chan et al., 2020). Strong causal evidence comes from studies showing that a similar pattern of offspring phenotypic changes emerge regardless of whether offspring are derived from natural breeding, *in vitro* fertilization, or intracytoplasmic sperm injection (Dias and Ressler, 2014; Wu et al., 2016; Chan et al., 2020; but see also Dietz et al., 2011). In order to implicate sperm miRNA content specifically in driving offspring outcomes, researchers have directly microinjected into zygotes miRNA from stress-exposed sperm or mimics of identified miRNA changed in stress-exposed sperm (Gapp et al., 2014, 2018; Rodgers et al., 2015). These experiments substantiate the claim that stress-altered sperm miRNA are responsible for transmitting offspring phenotypic changes.

How Stress May Alter sncRNA Content in Sperm

An important missing piece of the puzzle has been understanding how paternal stress leads to changes in sperm sncRNA content when sperm are transcriptionally inert. Recently, however, evidence has emerged that illuminates a potential mechanism. Epididymal epithelial cell extracellular vesicles in the caput epididymis encode environmental challenges through changes in their sncRNA content. As sperm transit through the epididymis during maturation, they gain sncRNA from extracellular vesicles (Belleannée et al., 2013; Nixon et al., 2015; Sharma et al., 2016). The current understanding is that the somatic epididymal epithelial cells maintain epigenetic changes following stress and that this affects the sncRNA content of secreted extracellular vesicles. Thus, these cells are able to encode and signal stress to

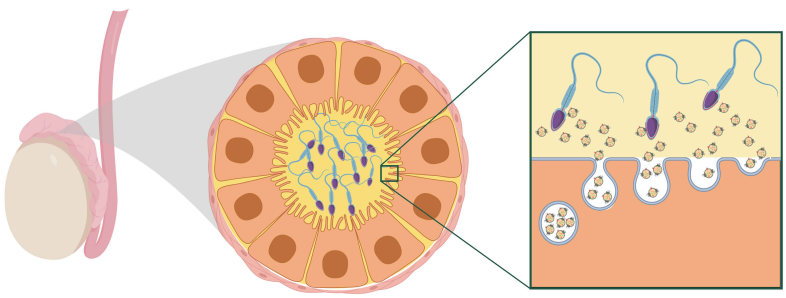

FIGURE 2 | Hypothesized model of maturing sperm interacting with extracellular vesicles within the caput epididymal lumen. Reprinted from Chan et al. (2020). Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. Nature Communications. 11:1499. https://creativecommons.org/licenses/by/4.0/.

sperm even after the stress exposure subsides (Morgan et al., 2019; see **Figure 2**).

A recent study in mice provides some of the first evidence that extracellular vesicles secreted from epididymal epithelial cells are able to transmit stress signals to sperm and reproduce a specific offspring phenotype of paternal stress (Chan et al., 2020). Epididymal epithelial cells in culture were chronically treated with either corticosterone or vehicle for three days, and extracellular vesicles recovered from the media of these cells were collected 8 days later, following three media changes occurring after treatment. Sperm collected from stress-naïve male mice were incubated with extracellular vesicles from either the corticosterone or vehicle treatment. Sperm were then injected into control oocytes via intracytoplasmic sperm injection and transferred into the right or left uterine horn of recipient females to control for the intrauterine environment. In embryos derived from sperm exposed to corticosterone-treated extracellular vesicles, the developing brain at mid-gestation showed significant changes in genes underlying synaptic signaling and neurotransmitter transport. As adults, offspring generated from the corticosterone-treated extracellular vesicles phenocopied the blunted corticosterone response of offspring produced from naturally bred stress-exposed males. Importantly, this study provides evidence for somatic to germ cell transmission of a stress signal. In this model, exposure to stress is signaled in an existing signaling pathway along the reproductive tract. This mechanism promotes lasting programmatic change in a way that allows the paternal environmental experience to be transmitted between generations but does not itself require perpetuation of an epigenetic mark following conception.

DISCUSSION

Evidence supporting epigenetic transmission of stress is still emerging, and many important questions remain. Although studies have found that paternal preconception stress is associated with epigenetic alterations in sperm, the value of these differences remains largely unknown. Even as some evidence has confirmed that epigenetic modifications identified in sperm alter offspring development in a meaningful way, the biological mechanism remains unclear for how these epigenetic changes impact offspring development. The prevailing thought is that sncRNA alter the developmental trajectory of the offspring by initiating a cascade of molecular events that propagate the stress signal, shifting the timing of developmental events and guiding development toward a set of neurobiological, physiological, and behavioral traits that are more adaptive in a stressful environment (Morgan et al., 2019, 2020; see **Figure 3**).

Sperm sncRNA play a critical role in early zygote and embryo development (e.g., Liu et al., 2012; Sendler et al., 2013; Yuan et al., 2016; Guo et al., 2017; Conine et al., 2018; Godia et al., 2018). Even relatively minor modifications in sperm sncRNA content could shift timing of developmental

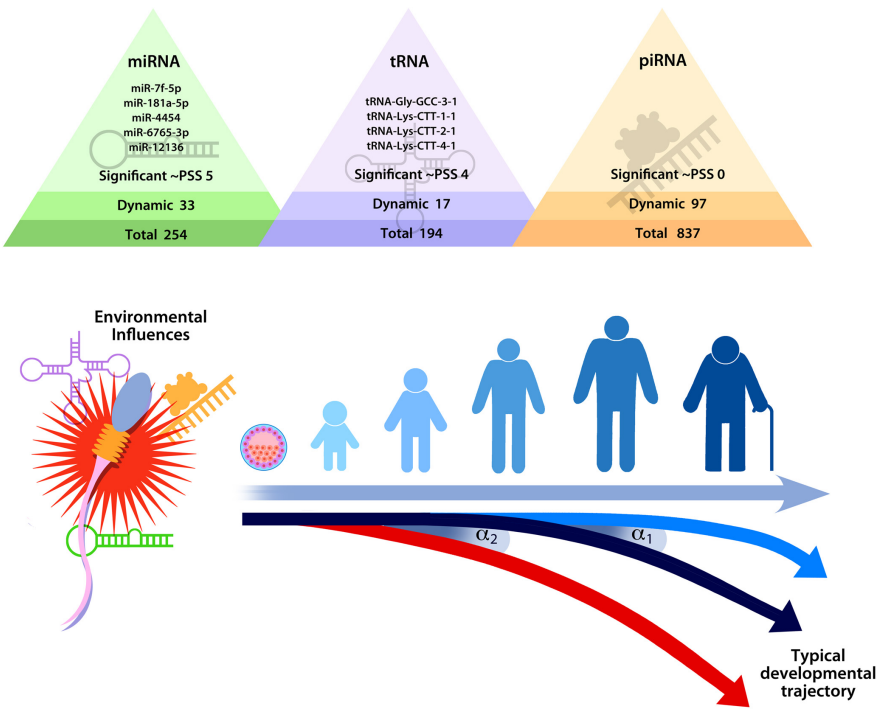

FIGURE 3 | Hypothesized model of the impact of stress on sperm small non-coding RNA content on developmental trajectories, highlighting the fact that relatively minor changes in sperm small non-coding RNA (as indicated by α1 or α2) could shift the timing of developmental events (e.g., embryo division and implantation) in ways that produce significant differences over time. Reprinted from Morgan et al. (2020). Repeated sampling facilitates within- and between-subject modeling of the human sperm transcriptome to identify dynamic and stress-responsive sncRNAs. Scientific Reports. 10:17498. https://creativecommons.org/licenses/by/4.0/.

events in a meaningful way that could produce significant phenotypic changes in the offspring. A feasible example of how sncRNA might affect development comes from consideration of protogenin, a gene that regulates early embryonic transitions (Wong et al., 2010; Chen and Wang, 2020). Two of the stress-responsive sncRNA identified in human sperm target protogenin (Morgan et al., 2020). Therefore, it is conceivable that changes in the levels of sperm let-7f-5p and miR-181a-5p at conception could dynamically regulate protogenin expression and impact the rate of zygotic and embryonic division. If stress-induced epigenetic modifications delivered by sperm indeed affect the rate of zygotic and embryonic development, then this would fit within the framework established by previous research. Specifically, early life stress effects on developmental programming that prepare the offspring for adverse conditions may originate preconception.

Though the cascade of developmental changes that result from the epigenetic signal may be intended to be adaptive, these phenotypic changes may nevertheless have implications for risk and resilience to disease in the offspring. However, it is important to emphasize that epigenetic changes are not deterministic, and the majority of the studies to date only show "differences," not a disease. Epigenetic effects on the offspring may tip the scale toward risk or resilience, but the emergence of disease relies on the interplay between genetic and environmental risk factors.

More studies are needed before firm conclusions can be drawn. For research in females, strong methodologies are needed to contend with critical confounds – namely the intrauterine environment and maternal behavior. For example, in humans, one study showed that the intrauterine environment can transmit maternal preconception stress to offspring via greater placental corticotropin-releasing factor during pregnancy (Moog et al., 2016). In animal studies, transferring the embryo to a surrogate female is a powerful method that allows researchers to disentangling preconception effects on oocytes from effects *in utero* (Tsai et al., 2020). Likewise, preconception stress in rats affects the quality of maternal behavior, another potential confounding factor (Keller et al., 2019). Cross-fostering newborns with a surrogate mother allows biologically driven effects of maternal preconception stress to be separated from socially driven effects.

Broadly, future research must consider important moderators in order to draw conclusions that are more nuanced rather than oversimplified. Almost no research has examined whether the type and timing of the stressor is critical to alterations in the offspring phenotype. In the current literature, the impact of stressor type and timing is difficult to disentangle because studies use different stress paradigms, assess different outcomes, and do not always specify the developmental time period of stressor exposure. To our knowledge, only one study has examined the impact of developmental timing within the same experiment (Rodgers et al., 2013). Although this study did not observe phenotypic differences in the offspring of male mice depending on whether they were exposed to stress in adolescence or adulthood, one study in humans suggests that developmental timing may matter (Bygren et al., 2001). In this retrospective study, nutritional challenges during the grandfather's preadolescent slow growth phase were associated with changes in the grandson's longevity. This finding suggests that future studies should consider the impact of stressor type and timing. In addition, many studies report sex differences, suggesting that parent and infant sex may be important moderators of epigenetic germline transmission of stress and that future research should test for sex differences (Shachar-Dadon et al., 2009; Franklin et al., 2010; Saavedra-Rodríguez and Feig, 2013; Bohacek et al., 2015; Zaidan and Gaisler-Salomon, 2015; Bock et al., 2016; Wu et al., 2016; Cissé et al., 2020). Finally, prospective longitudinal studies are needed in order to translate hypotheses generated in animal models to humans. Only then will we be able to differentiate the effects of different types of real-life stress exposures on germ cell epigenetic changes in humans.

AUTHOR CONTRIBUTIONS

KD drafted the manuscript under the guidance of TB and CNE. TB and CNE provided critical revisions and feedback. All authors contributed to the article and approved the submitted version.

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The remaining 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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Maternal Parenting Stress in the Face of Early Regulatory Disorders in Infancy: A Machine Learning Approach to Identify What Matters Most

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Objective: Early regulatory disorders (ERD) in infancy are typically associated with high parenting stress (PS). Theoretical and empirical literature suggests a wide range of factors that may contribute to PS related to ERD. The aim of this study was to identify key predictors of maternal PS within a large predictor data set in a sample of $N = 135$ mothers of infants diagnosed with ERD.

Methods: We used machine learning to identify relevant predictors. Maternal PS was assessed with the Parenting Stress Index. The multivariate dataset assessed cross-sectionally consisted of 464 self-reported and clinically rated variables covering mother-reported psychological distress, maternal self-efficacy, parental reflective functioning, socio-demographics, each parent's history of illness, recent significant life events, former miscarriage/abortion, pregnancy, obstetric history, infants' medical history, development, and social environment. Variables were drawn from behavioral diaries on regulatory symptoms and parental co-regulative behavior as well as a clinical interview which was utilized to diagnose ERD and to assess clinically rated regulatory symptoms, quality of parent–infant relationship, organic/biological and psychosocial risks, and social–emotional functioning.

Results: The final prediction model identified 11 important variables summing up to the areas maternal self-efficacy, psychological distress (particularly depression and anger-hostility), infant regulatory symptoms (particularly duration of fussing/crying), and age-appropriate physical development. The RMSE (i.e., prediction accuracy) of the final model applied to the test set was 21.72 ($R^2 = 0.58$).

Conclusions: This study suggests that among behavioral, environmental, developmental, parent–infant relationship, and mental health variables, a mother's

higher self-efficacy, psychological distress symptoms particularly depression and anger symptoms, symptoms in the child particularly fussing/crying symptoms, and age-inappropriate physical development are associated with higher maternal PS. With these factors identified, clinicians may more efficiently assess a mother's PS related to ERD in a low-risk help-seeking sample.

Keywords: early regulatory disorders, machine learning algorithms, parenting stress, parental self-efficacy, infant mental health diagnostic

INTRODUCTION

Early regulatory disorders (ERD), which include sensory, sleeping, crying, or feeding disorders, are found in 10.9% of infants/toddlers and are among the most prevalent diagnoses in children under the age of four (1). The disorders have been repeatedly found to be associated with high parenting stress (PS) and parental burden (2, 3). Research has primarily focused on the effects of excessive crying and infant colic on parents: e.g., compared to control groups, mothers reported higher negative affect in response to the cries (4) and felt more sad and aroused by the cries (5). According to the developmental system model of ERD, the parental stress response to infants' regulation problems may contribute to a vicious circle of negative contingency that perpetuates parental burden, impairs parental self-efficacy, and leads—in the context of parent and child-related risk and protective factors—to the manifestation or perpetuation of ERD (6). Contrarily, in the context of a lower distress response of parents and through being more effectively co-regulated, infants' self-regulatory competence may increase.

Research showed that while ERD likely have far-reaching consequences for a child (7), they do not necessarily have such effects, but may be mediated by parents' burden. Smarius et al. found that the maternal burden of infant care partially mediated the association between ERD and later mood and behavioral problems in childhood (8). In addition, the level of maternal PS predicted the persistence of regulation problems (9). These studies suggest that reducing PS may be an effective objective in treating ERD and may also contribute to better long-term outcomes.

While the adverse effects of ERD on parents have been well-established, the specific risk and protective factors associated with PS when raising infants with ERD have yet to be explored. A set of risk factors for ERD have been proposed (6, 10), many of whom may play a role in parents' propensity to experience PS related to ERD: high prenatal maternal stress or lifetime depressive or anxiety disorders have been found to predict ERD (11, 12), and socio-demographic risk factors, such as low social support or low maternal education, have been shown to be related to ERD (9, 11, 13) and may negatively affect PS (14, 15). PS has also been linked to miscarriages or abortions (15), which have been found to be more prevalent in a clinical ERD sample (6). Peripartum risk factors, like complicated pregnancy or birth which are more frequent in ERD samples (6, 16), may affect parents' perception of infants' distress and thus their propensity to experience PS. Other infant diagnostic characteristics, such as the presence of an organic condition or difficult temperament, were related to ERD (6, 13) and may increase PS (17). Maternal self-efficacy was a protective factor for reporting ERD (11, 18) and may protect against high PS. Similarly, parental reflective functioning may be a protective factor for high PS related to ERD (19).

While the existing body of literature provides valuable data on distinct predictors and correlates of ERD, the extent to which these variables are associated with maternal PS (MPS) in the context of ERD has rarely been investigated. An additional limitation of the literature is the inclusion of a small number of variables, despite multiple and interrelated factors within a family system that may relate to PS. Thus, clinicians wishing to help families who experience ERD by reducing parental burden of infant care must assess a vast number of possible variables that may be related to the PS. Furthermore, few of the studies included samples of infants with ERD beyond excessive crying. However, most of the help-seeking parents report multiple regulatory problems (3, 13) and there is a need to understand more about this group. Thus, the goal of this study was to identify key variables associated with MPS in ERD by utilizing a large predictor variable set. To this end, machine learning (ML) was applied.

ML approaches for clinical psychology and psychiatry perform statistical functions on multidimensional data sets to make generalizable predictions about individuals, e.g., they can be used to provide predictive models for diagnostic classification or treatment response of individual cases. In the field of child psychiatry, ML may prove especially useful for the incorporation of data from different sources like behavior measures, genetics, and environmental as well as developmental factors (20). This advantage is a product of ML being able to integrate large sets of correlated variables while assuming no distribution in the outcome or underlying data mechanism and being largely insensitive to outliers (21). In addition, prediction models can include data on single-item level. For example, Carpenter et al. tested whether individual diagnostic interview items from the Preschool Age Psychiatric Assessment predicted with high reliability and low false-positive rates whether a child is likely to have an anxiety disorder (22). The resulting variable importance ranking can be utilized to shorten diagnostic batteries for more effective diagnostics and for identifying cases that may need further evaluations. All of these features are especially useful for the field of infant mental health research where multiaxial diagnostics are the norm.

We employed ML in an exploratory search for variables best predicting a mother's MPS related to ERD in a cross-sectional study design. Predictor variables were empirically and theoretically derived (6, 10) and covered risk and protective factors as well as correlates that have been identified for ERD or PS. We included a multivariate dataset by utilizing multiple measures covering self-report and clinical ratings: mother-reported general psychological distress, maternal self-efficacy, parental reflective functioning, socio-demographic variables, each parent's history of illness, recent significant life events, former miscarriage/abortion, pregnancy, obstetric history, infants' medical history, development, and social environment. Behavioral diaries were used to assess infants' regulatory symptoms and extent of parental co-regulative behavior. We used all items from a structured clinical interview that was utilized to diagnose ERD and for the clinical assessment of regulatory symptoms, quality of the parent–infant relationship, organic/biological and psychosocial risks, and social-emotional functioning. In addition to global scores obtained from the instruments, we included all items gathered in our dataset on single-item and subscale levels in an effort to maximize specificity of the predictors. ML enabled us to analyze this large number of potential predictors simultaneously.

MATERIALS AND METHODS

Data were acquired from an RCT on the effectiveness of brief parent–infant psychotherapy for ERD, where data collection was still ongoing by the time of this study. We used baseline data gathered pretreatment at one time point. Data were collected from February 2014 to May 2017 in the department of Family Therapy at Heidelberg University Hospital.

The approval for research in this sample was obtained from the Ethical Committee of the Medical Faculty of Heidelberg University (approved in November 2013).

Participants

Families were referred from pediatric practices for the purpose of study participation if parents reported significant crying, sleeping, or feeding difficulties. Some families self-referred in response to public advertisement, websites, and flyers/posters distributed in gynecological, pediatric, and osteopathic practices, parent–infant groups, and crèches. All families participated in the RCT and were randomized to treatment conditions after data collection.

Inclusion criteria required the infant to be between 4 and 15 months old, to be born at full term (>37 weeks of gestation), and to meet diagnostic criteria for sleeping disorders, feeding disorders, or regulation disorders of sensory processing according to DC:0-3 R (23) or for persistent excessive crying, sleeping, and feeding disorder, according to the guidelines recommended by the German Society of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy (AWMF guidelines; AWMF No. 028/028) (24). Pregnancy needed to be singleton, and primary caregivers needed to speak German.

Participants were excluded when infants had a medical diagnosis that better explained the regulatory problems, a tentative diagnosis of fetal alcohol syndrome, or a diagnosed disability or developmental disorder. A very high symptom severity of the primary caregiver (Symptom-Check-List-90R-S, Global Severity Index of $T > 70$) also led to exclusion (25), as a current mental illness of the caregiver was considered to be a contraindication for the brief intervention given to the families within the RCT.

A total of 165 primary caregivers expressed their interest in study participation and underwent screening for eligibility via telephone. Parents were informed about the study and invited for participation if they consented. Of these, 24 canceled or did not show up. Six families fulfilled exclusion criteria and thus were excluded. The primary caretaker was asked to participate, which in all cases was the mother. The final sample consisted of $N = 135$ mother–infant dyads.

Procedure and Assessments

Self-report measures and behavioral diaries were mailed to mothers following the phone screen. Clinical diagnostics were led by two psychologists. The assessment was conducted with mother and infant and included the clinical interview and video recording of standardized parent–infant interactions. Written informed consent was gathered at the beginning of the session. Clinical ratings were performed immediately after the interview.

Maternal Parenting Stress

The Parenting Stress Index (26) assesses self-reported PS with 48 items. Items are rated on a five-point Likert scale from 1 (*strongly disagree*) to 5 (*strongly agree*). Higher scores indicate higher PS. Items are summed up into one global score. Cronbach's α was excellent in this study (0.94).

Psychological Distress

The Symptom-Checklist (Symptom-Checklist-90R-S, SCL) assesses self-reported psychological symptoms and psychopathology (25). The 90 items are rated on a five-point Likert scale from 0 (*not at all*) to 4 (*extremely*) with higher scores indicating higher distress. Items add up to 10 subscales (somatization, obsession–compulsion, interpersonal sensitivity, depression, anxiety, anger–hostility, phobic anxiety, paranoid ideation, psychoticism, and additional clinical symptoms), a sum score, and the global severity index (GSI). Cronbach's α was between 0.56 (psychoticism) and 0.84 (obsession–compulsion) and was excellent for the sum score (0.96).

Maternal Self-Efficacy

The Maternal Self-Efficacy Scale (MSES) assesses self-reported behavioral competence in parenting (27). For this study, back-translation procedures were implemented, and the final version was reviewed by an English native speaker. The 10 items are rated on a four-point Likert scale from *not good at all* (1) to *very good* (4). Cronbach's α was acceptable (0.75).

Parental Reflective Functioning

The Parental Reflective Functioning Questionnaire (PRFQ) uses 18 items in order to assess the scales (28): (a) interest and

curiosity in mental states (IC), (b) certainty of mental states (CMS), and (c) prementalizing (PM). Items are rated on a seven-point Likert scale ranging from *strongly disagree* (1) to *strongly agree* (7). Cronbach's α was acceptable for CMS (0.73), poor for PM (0.57), and unacceptable for IC (0.47).

Parent-Questionnaire

The Parent-Questionnaire (29) was developed for the comprehensive data assessment of parents and their children with ERD. Questions refer to the areas socio-demographic information, history of illness, recent significant life events, former miscarriage/abortion, pregnancy, obstetric history, and infant medical history, development, and social environment. Variables are assessed dimensionally and categorically or in open format; no sum scores are provided. For the analysis, 110 single items were used (see **Supplemental Material** and **Supplementary Table 1**, which lists the items per area).

Clinical Interview

A structured clinical interview was developed to assess axis I (DC:0-3R) (23) on sleep-onset disorder, night-waking disorder, feeding disorders, and regulation disorders of sensory processing. Persistent excessive crying syndrome is not mentioned as a clinical category in DC:0-3R, and diagnostic criteria are poorly described (23). Therefore, we additionally utilized the AWMF guidelines on persistent excessive crying, sleep-onset disorder, night-waking disorder, feeding disorders, and pervasive regulatory disorder (AWMF) (24). The parent–infant relationship global assessment scale (PIR-GAS, DC: 0-3R) dimensionally assesses the parent–infant relationship from *documented maltreatment* (0–10) to *well adapted* (91–100). Medical conditions of the infant (axis III of DC:0-3R) and psychosocial stressors (axis IV of DC:0-3R) were dimensionally assessed using organic/biological and the psychosocial risk scales (30). Infants' emotional and social functioning (axis V of DC:0-3R) was rated on the proposed rating scale (DC:0-3R). In sum, 150 variables covering single symptoms, sum scores of symptoms on the level of diagnosis and axis, and a general symptom sum score were used in analysis (see **Supplementary Table 1**).

Infant Regulatory Symptoms

The Questionnaire for Crying, Feeding, and Sleeping (QCFS) (31) assesses crying, sleeping, and feeding symptoms and parents' dysfunctional co-regulation behavior in response to the symptoms (e.g., "only falls asleep when being carried"). The 53 items constitute the three scales, (a) fussing/crying and sleeping, (b) feeding, and (c) dysfunctional co-regulation, and a global score. Higher scores indicate more symptoms, parental burden, and dysfunctional co-regulation. Frequency questions are rated on a four-point Likert scale from *never/rarely* (1) to *always/every day* (4). Parents' perceived difficulty is rated from *not at all* (1) to *a lot* (4). Cronbach's α is good for the scales (scale 1 = 0.82; scale 2 = 0.76; scale 3 = 0.84) and the global score (0.82).

96-H Behavior Diary

The diary of crying, sleeping, and feeding behavior (32) is similar to widely used parental diaries and assesses infants' behavior and parents' co-regulation behavior. Frequency and duration of fussing/crying, sleeping/waking, feeding, and parental co-regulation is recorded in 15-min intervals on four consecutive days. Additional questions refer to the success of parental co-regulative strategies. In sum, 139 variables were used in the analysis (see **Supplementary Table 1**).

Infant Development

The Ages and Stages Questionnaire (ASQ-3) is a series of 21 parent-rated questions on children's developmental performance in communication, gross motor, fine motor, problem solving, and personal–social skills, represented on five scales (33). The 30 items are rated with regard to the child's competence as *yes* (10), *sometimes* (5), or *not yet* (0). We used the German translation of the questionnaires for 4-, 6-, 8-, 9-, 10-, and 14-month-old infants. Internal consistency of the scales was not calculated, due to some small age-dependent subgroups. Other studies have shown it to be poor to excellent (33).

The PSI, SCL, and the QCFS are valid, reliable measures. For the MSES, PRFQ, and ASQ-3, validity and reliability have only been demonstrated for the original English version.

Statistical Analysis

Since ML algorithms do not assume a certain distribution underlying the data (i.e., normal distribution, binomial distribution), the common assumptions for classical parametric analysis like homoscedastic or normally distributed residuals do not apply and the analysis handles smaller sample sizes in relation to the number of variables.

For the prediction of the PSI, all data provided by questionnaires, behavioral diary, and clinical interview on the level of items, subscales, and global scores were used, resulting in 596 variables. Of these, variables with <50% missing values before imputation were used, resulting in a final set of 464 variables. The remaining data contained 5.48% missing values. Imputation was done assuming missing at random after visual inspection of pattern of missingness plots. Multiple imputations by chained equations (34), using fully conditional specification with 40 iterations, were utilized to produce asymptotically unbiased estimations of the data.

An important difference between ML approaches and more commonly used statistical methods is the absence of *p* values and, furthermore, in-sample model fit as a measure of "success." In ML, the main statistic of interest is the prediction accuracy which is why there are usually two phases: Training the algorithm and testing the result for generalizability. To this end, data in our study was split into a training set containing 70% of all cases and a test set containing the remaining 30%.

The general process of data analysis was carried out as follows. In the first phase, we trained our ML algorithm on the training set in order to select the best-performing algorithm. This was done by using Gradient Boosting Machines (GBM) with feature selection (FS). The algorithm was trained using 5-fold cross-validation and 10 repeats. In this procedure, the data is split into 5 folds (i.e., groups), and each fold is, in turn, left out of the training procedure and used to validate the results of training. The resulting accuracies are then averaged and provide a stable estimate of generalizability (21). In the case of the present study,

TABLE 1 | Sample characteristics of infants and their mothers ($N = 135$).

Variable	*M*/%	*SD*
Infant age (in months)	8.55	3.10
Mother age (in years)	33.27	4.47
Girls	45.2%	–
Firstborn child	65.2%	–
Mother has high school or higher education	74.8%	–
Mother married	79.3%	–
Mother of German origin	79.3%	
Mother with mental disorder lifetime	14.8%	–
Diagnoses		
Persistent excessive crying	8.9%	–
Regulation disorder of sensory processing	44.4%	–
Feeding disorder	13.3%	–
Sleeping disorder	95.6%	–
> 1 diagnoses	48.0%	–
PIR-GAS	74.96	9.76
SCL (GSI)	49.00	34.18
PSI	131.50	31.60

PIR-GAS, Parent–Infant Relationship General Assessment; SCL (GSI), Global Severity Index of the Symptom-Severity-Check-List-90R-S; PSI, Sum score of the Parenting Stress Index.

the data of the 95 participants that were randomly selected to be the validation set was first divided at random into 5 folds of 19 participants. Following, the algorithm predicted the PSI value of all participants in one of the 5 folds based on the data of the other 4 folds. The difference between the predicted PSI values in that fold and the observed PSI values was then computed and averaged (in our case, root mean square error, RMSE, as well as mean absolute error MAE were calculated) over all observations. A relatively low difference between the observed and predicted values is an indicator of good generalizability, while high differences indicate bad generalizability. This process was repeated with every fold, and the result was averaged over all iterations. This was then, in turn, repeated 10 times with different splitting points to form the folds of 19 participants for the data.

In the second phase, the so trained model was validated using the holdout data of the remaining 40 participants. Prediction accuracy was computed by comparing values predicted by the trained algorithm with the observed PSI values of the holdout sample in the same manner as described above.

All statistical analyses were performed with R version 3.5.2 (35). The R package "caret" version 6.0-76 was used to train the algorithm.

Feature Selection (FS)

FS is a procedure to select optimal variables from a larger data set with the aim of increasing predictive performance and is part of preprocessing the data. We utilized FS since our data covered a heterogeneous set of markers and we predicted that our variable set would have few variables with large predictive effects on the PSI and many variables with medium to small effects. We used a recursive backward selection, based on importance ranking of random forests out of the entire set of 464 variables. The procedure works by fitting a random forest model using all predictors and calculating the contribution of each predictor using the variable importance metrics. Following, starting from $S = 464$ and going down to $S = 1$, a similar random forest model is fitted using the S most important variables calculated before and the performance of the model is tracked. Based on the performance profile of all models S464 through S1, the appropriate number of predictors for the final model was determined by using the predictors of the best-performing model.

The result was a set of 50 variables that were deemed to be most informative in terms of PSI and which were used in combination with the algorithm as described below. The FS "rfe" function from the caret package was used to implement this.

Gradient Boosting Machines (GBM)

For the final model following the feature selection, we used GBM which is suited for data with established features and is relatively easy to tune. The GBM was tuned by a hyperparameter grid search for the optimal model parameters, which was done by iteratively manipulating the shrinkage coefficient (eta) between 0.01 and 0.2, the interaction depth of each tree (max_depth) between 1 and 6, and the number of boosting iterations (nrounds) between 1 and 1500, while keeping the minimum loss reduction (gamma) fixed at 0 and the minimum sum of instance weight (min_infant_weight) fixed at 1. This represents a conservative approach with low likelihood of overfitting. The final values for the model were eta = 0.01, nrounds = 500, max_depth = 1. The gradient boosting model "xgbTree" from the caret package was used.

In an effort to rank the predictors of the PSI according to their importance or contribution for predicting PSI scores, we analyzed the variable importance of the final GBM model. This was done by summing the relevance for each predictor variable for each internal node of the tree for which the predictor was chosen as a splitting variable. Since this measure is relative, the most important variable was assigned the value of 100 while the others were scaled accordingly.

After the most relevant variables had been identified, the next step was to assess the marginal effects in which each of the predictor variables influenced PSI. Therefore, we examined partial dependency plots of the most important variables and their interrelation in predicting PSI. Marginal effects were calculated using Friedman's tree traversal method (36).

RESULTS

Participants

Table 1 gives an overview of the sample characteristics. On average, the parent–infant relationship was rated as perturbed (PIR-GAS, 71-80). The percentage of maternal lifetime mental illness was lower compared to the lifetime prevalence rates in Germany (25.2%) (37). Mothers' average psychological distress (SCL-GSI) was equivalent to a *T*-score of 57, which is $\sim$ >1 *SD* higher compared to the normative sample (25). On average,

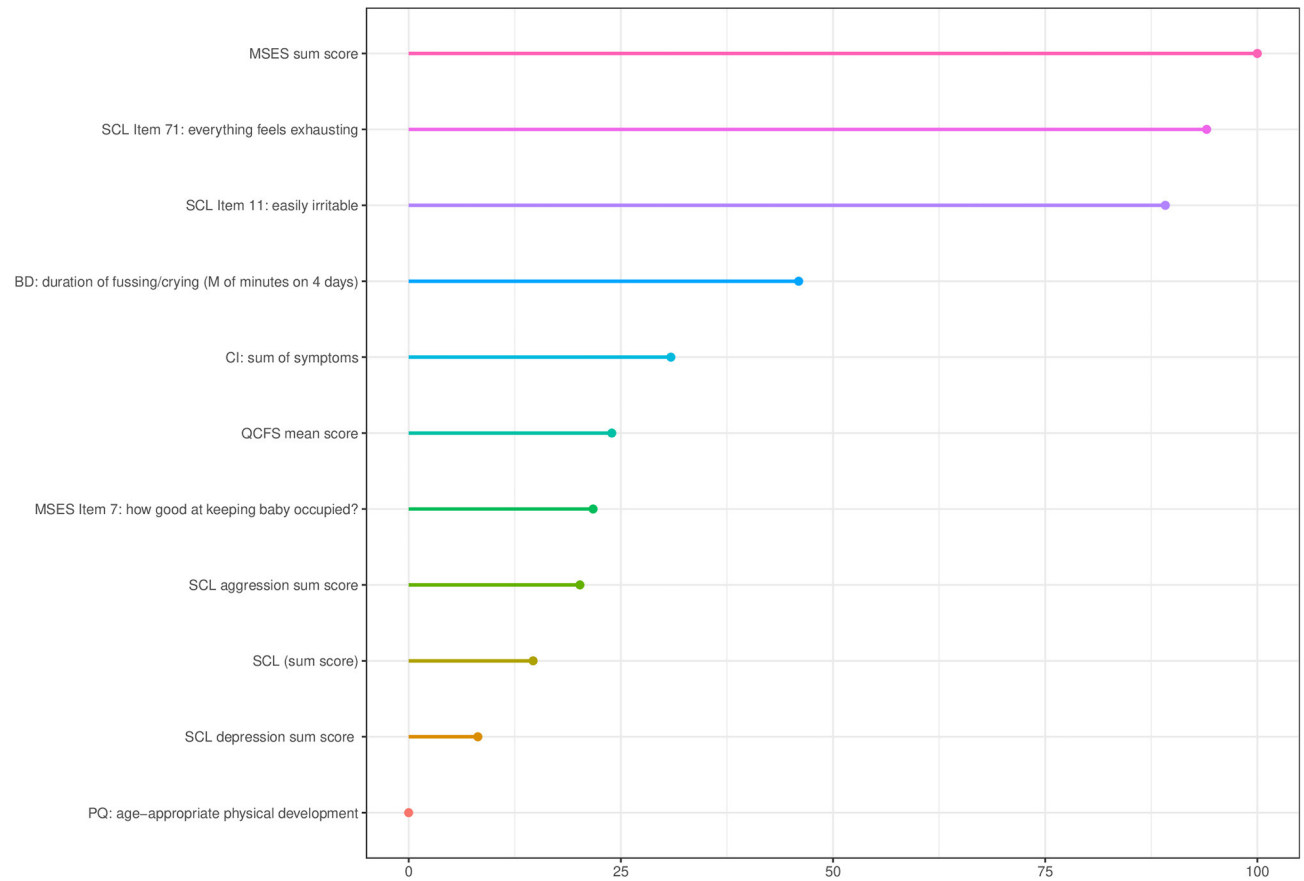

FIGURE 1 | Relative importance of variables in predicting PSI extracted from the tuned model. CI, Clinical interview; MSES, Maternal Self-Efficacy Scale; QCFS, Questionnaire for Crying, Feeding, and Sleeping; SCL, Symptom-Severity-Check-List-90R-S; PQ, Parent-Questionnaire; PSI, Parenting Stress Index; BD, 96-hour behaviour diary.

TABLE 2 | Descriptive statistics of the PSI outcome and the top 11 most important variables for the prediction of PSI.

Variable	*M* (%)	*SD*	*Mdn*	min	max	Range
Outcome (PSI sum score)	131.50	31.60	131	59	218	159
BD: duration of fussing/crying (M of minutes on 4 days)	189.02	140.88	168.75	11.25	937.5	926.25
CI: sum of symptoms	12.20	5.98	12	3	35	32
MSES sum score	31.7	3.62	31	21	40	19
MSES Item 7: how good at keeping baby occupied?	2.55	0.84	3	1	4	3
PQ: age-appropriate physical development	0.97	0.24	1			2
0 (no)	6 (4.44)					
1 (yes)	127 (94.07)					
2 (uncertain)	2 (1.48)					
QCFS global score	2.21	0.22	2.22	1.63	2.83	1.21
SCL (sum score)	49.00	34.18	41	0	159	159
SCL anger–hostility subscale	4.45	4.42	3	0	20	20
SCL depression subscale	10.99	8.11	9	0	43	43
SCL Item 11: easily irritable	2.05	1.22	2	0	4	4
SCL Item 71: everything feels exhausting	1.70	1.30	2	0	4	4

CI, clinical interview; MSES, Maternal Self-Efficacy Scale; QCFS, Questionnaire for Crying, Feeding, and Sleeping; SCL, Symptom-Severity-Check-List-90R-S; PQ, Parent-Questionnaire; PSI, Parenting Stress Index; BD, 96-h behavior diary.

they experienced more MPS (PSI) than 88% of the normative sample (26).

Performance

The RMSE (i.e., prediction accuracy) of the final model applied to the test set was 21.72, the R^2 was 0.58, and the MAE was 17.04. Thus, the algorithm on average over- or underestimated the observed PSI score of the participants by 17.04 points or within 10.72% of the observed PSI range which was 159. Thus, by using the final model, an individual mothers' MPS can be predicted within the range of 17.04 points on the PSI score which equals about half a standard deviation on the observed PSI range.

The relatively small difference between RMSE and MAE indicates that there were few observations that had larger than average residuals. This indicates that the model's predictive performance is independent of the observed PSI scores (i.e., the model predicts equally well - irrespective of high, low, and medium PSI scores of the participants).

Importance of Variables

Figure 1 displays the relative importance of the variables in predicting PSI. The 11 variables relatively contribute more to the prediction of mothers' MPS compared to the remaining 50 variables. After inspecting the importance graph of the final model, the most important variables were chosen since there was a large gap in variable importance compared to the rest after the top 11.

Table 2 shows the descriptive statistics of the top 11 important variables. Among the most important predictors were maternal self-efficacy (MSES sum score) and two items of the SCL-90R-S that assess exhaustion (item 71) and irritability (item 11).

Partial Dependency Plots

The partial dependency plots display the individual contribution of each of the 11 most important variables to the prediction of the PSI score. Additionally, we examined the interrelations of the four most important variables in predicting MPS.

Figures 2, **3** show the marginal effect of the MSES sum score together with either item 71 of the SCL (everything feels exhausting) or the duration of fussing/crying documented in the behavioral diaries. In both figures, a plateau effect of MSES can be observed, where values lower than 31 or higher than 34 have little effect. In addition, **Figure 2** shows a plateau effect for SCL-90R-S Item 71: Values below the sample mean of 1.7 are indicative of low MPS while values above 1.7 are indicative of higher PS.

Figure 3 shows a linear increasing effect of the duration of fussing/crying on MPS up until 500 min (8.33 h per day) while the plot slightly dips afterward and only five participants reported values above 500 min.

Partial dependency plots on the relation between all important variables, and the PSI score are provided in the supplement (**Supplementary Figures 1–11**).

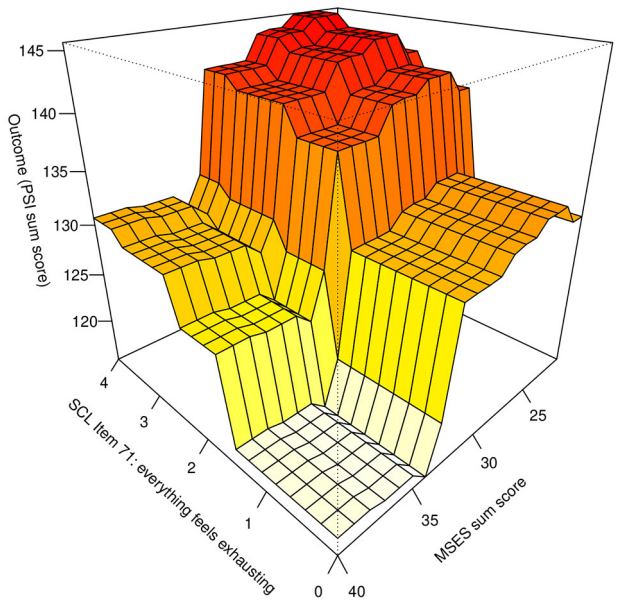

FIGURE 2 | Marginal effect of MSES sum score together with the SCL Item 71 on predicted PSI value. MSES, Maternal Self-Efficacy Scale; SCL, Symptom-Severity-Check-List-90R-S; PSI, Parenting Stress Index.

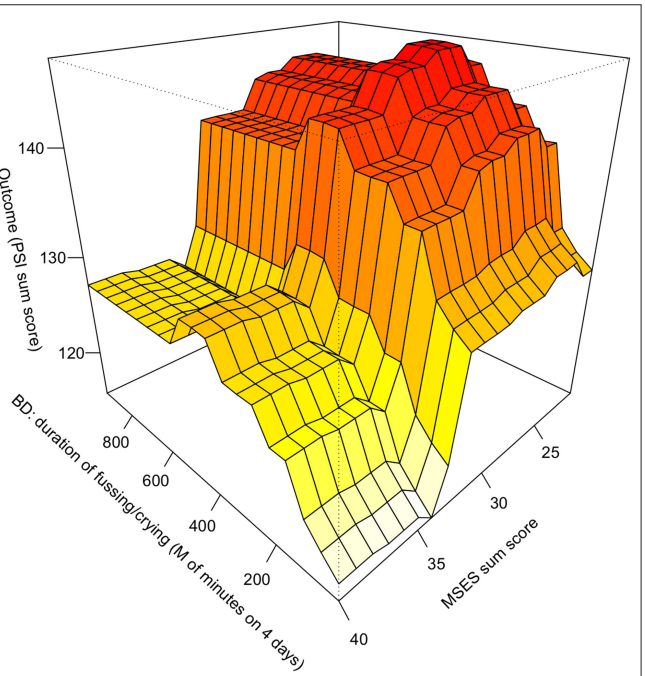

FIGURE 3 | Marginal effect of MSES sum score together with the duration of fussing/crying (BD) on predicted PSI value. BD, 96-hour behaviour diary; MSES, Maternal Self-Efficacy Scale; PSI, Parenting Stress Index.

DISCUSSION

To the best of our knowledge, this study is the first to have explored factors related to MPS in ERD by including a range of parent- and infant-related variables like behavioral, environmental, developmental, parent–infant relationship, and mental health variables. We used an ML approach as the

variables of interest included a multitude of differentially scaled and potentially correlated variables that may have non-linear associations with PS and because ML enabled the use of predictor variables on single-item level in order to maximize specificity in the prediction models. As expected, mothers in our sample reported much higher average MPS compared to a normative sample, while there was a high range on the PSI score pointing to strong interindividual differences in how stressful mothers experienced parenting their child. Upon analysis of 464 variables involving self-report questionnaires, behavioral diaries, and clinical assessments, we found 11 most important predictors for MPS that can be summed up to the following areas: maternal self-efficacy, psychological distress (particularly depression and anger–hostility), infant regulatory symptoms (particularly fussing/crying), and age-appropriate physical development.

While the majority of these predictor and correlating factors support existing literature on ERD or PS in general, this study highlights their relative importance compared to other possibly relevant parent- and infant-related variables that were included and helps to specify which items particularly were relevant. Overall, our results demonstrate that a higher level of MPS in ERD was mostly associated with lower self-efficacy, stronger psychological distress symptoms particularly depression and anger symptoms, and a high degree of symptoms in the child particularly fussing and crying while the impression of the lack of an age-appropriate development was least important among the top predictors. Thus, the current psychological situation of the mother–infant dyad majorly accounted for MPS, while distal risk and protective factors were less important.

Utilizing cross-validation, we found that the model would likely generalize well to a similar population. The identified key variables can be used to select help-seeking mothers who are at an increased risk for experiencing high MPS in order to further evaluate these cases and to guide treatment of ERD. Approaching the identified factors in treatment may exert a positive effect on a mother's PS and thus on the negative reciprocities known to maintain or worsen ERD (6). Below, we discuss the important variables and implications of our results in detail.

Maternal Self-Efficacy

The maternal self-efficacy sum score (MSES) was the most important predictor in the final model and was—as expected—negatively related to PS. The relative importance of the construct for ERD is in line with previous research: compromised maternal self-efficacy has been described as an important factor in the etiology or perpetuation of ERD (6), while higher self-efficacy may be ameliorative to PS (18). Although mothers in our sample on average rated themselves as "good enough" in terms of how effective they experienced themselves across different parenting situations, the range in this scale was broad (**Table 2**) with the observed minimum of 19 points being equivalent to a rating of "not good enough." Mothers with such low expectations were prone to experience high MPS. Our research demonstrates, that in the face of ERD, mothers with low maternal self-efficacy experience high MPS and vice versa.

In addition, we identified incremental effects between low MSES scores and either exhaustion (SCL-90R-S item 71, **Figure 2**) or duration of infant fussing and crying (behavioral diary, **Figure 3**). This means for example that if a mother reported low self-efficacy in addition to experienced considerable exhaustion or experienced $\geq$3 h of fussing/crying per day, the model predicted significantly more MPS compared to mothers who did not fit these criteria. Thus, each of these factors combined seem to play a major role for MPS or vice versa. This result speaks for a cumulative effect where lower self-efficacy is negatively associated with MPS which gets worse the more exhausted the mother is and the more the child fusses/cries.

In addition, our results highlight a specific aspect of maternal self-efficacy important for ERD—the self-efficacy mothers experience when successfully occupying their infant: The MSES item "good at keeping baby occupied" had an additional, albeit less important role in the prediction. On average, mothers reported comparably lower self-efficacy regarding this specific parenting situation in contrast to the mean of the MSES (**Table 2**). This item might be especially relevant because occupying the child is a parenting task that continually arises throughout the day. Low expectations with this regard seem to be especially relevant to maternal parenting stress levels.

These results have several implications. Given the importance of maternal self-efficacy related to MPS in ERD, clinicians should assess and be aware of its deviations in order to align interventions. The combination of low self-efficacy with either high amounts of infant fussing/crying or high exhaustion of the mother should be especially considered when identifying and treating samples with ERD who target MPS. We identified a subgroup of mothers who reported high self-efficacy who experienced less MPS, despite the challenging conditions they faced. Higher self-efficacy may help in coping with prolonged fussing/crying but also in coping with exhaustion. Future research may focus on this subgroup to investigate conditions under which maternal self-efficacy can be a protective factor for MPS. This result also hints to a potential heterogeneity of etiological or maintaining factors for ERD.

Mothers' Psychological Distress

The second area of predictors was maternal psychological distress symptoms experienced during the last week, as was reflected in the two subscales depression and anger–hostility and independently two items from these subscales (exhaustion and irritability), and in the SCL-90R-S sum score, which were all positively associated with the PSI. Surprisingly, the two single items were among the three most important predictors in the dataset. The partial dependency plots further specified nearly linear relations between mothers' exhaustion and irritability with the PSI score (see **Supplementary Figures 10**, **11**). We noticed that mothers in our study compared to a normative sample were more psychologically distressed on average while displaying a high range on the SCL-90R-S sum score. T-values of the subscales depression ($T = 60$) and anger/hostility ($T = 62$) indicated a noticeable higher distress in these domains (25), suggesting that these are specific vulnerability factors in our sample.

Our results add to the notion that parents who are more depressed experience parenting in ERD as more difficult (38) and moreover specify which emotional aspect of depression is especially relevant to MPS in ERD. Accordingly, more exhausted mothers experience parenting as even more stressful compared to less exhausted mothers. It is also likely that depressive symptoms and anger–hostility inhibit parenting skills and thus increases MPS, given the studies showing that symptoms are linked to parenting impairments (39). Meanwhile, it is also plausible that a mother, who experiences more difficulties in parenting, reactively develops symptoms of depression and irritability as a result of helplessness and a lack of self-efficacy. Drawing from our results, clinical assessments and treatment conceptualization for ERD may especially consider these specific psychological distress symptoms of mothers.

While we found that current psychological distress symptoms, which in case of higher scores on the SCL-90R-S sum score point to a possible mental illness, were an important predictor for MPS, maternal lifetime mental illness was not among the critical variables. This result aligns with studies showing that PS was unrelated to prenatal anxiety or depression in no-risk infant samples (15) and may indeed play a subordinate role in parental burden related to ERD (40). However, several aspects need to be considered when interpreting our results: mothers with severe psychological distress, which increases the probability of a current acute mental illness, were excluded from study participation. Additionally, since we utilized only self-report measures, lifetime mental illness may have been underreported (41). Both of these factors may have contributed to the low prevalence rate of mental illness, thereby reducing the likelihood to generate meaningful results. Future studies should assess a more representative parent sample utilizing interview-based measures in order to clarify this question.

Infants' Regulatory Symptoms

Three variables indicative of infants' regulatory symptoms were important in predicting PS: the duration of fussing/crying as documented by mothers in behavioral diaries, the amount of clinically assessed regulatory symptoms (sum of symptoms in the interview), and the QCFS sum score which reflects general infant regulatory symptoms severity. As expected, all variables were positively related to MPS.

Behavioral observations of prolonged fussing and crying came up as the fourth most important variable in our dataset. The importance of this variable in the prediction, as opposed to other ERD symptoms, was particularly unexpected, as only 8.9% of infants were diagnosed with persistent excessive crying disorder. Given the high prevalence rates of sleeping disorders in our sample, it would have been plausible that sleeping symptoms (e.g., number of times waking up during the night) stood out in the prediction of PSI scores. The descriptive statistics indicate an overall high level of combined fussing and crying times with a mean of over 3 h and a maximum of 15.39 h per day (**Table 2**). Although values >8.33 h per day were infrequent, this result is in itself an important contribution to the literature and warrants further investigation. One possible explanation for the high prevalence in our sample is that different ERD are likely related to fussing and crying. For example, difficult sleep–wake regulation has been associated with difficult temperament and low sensory thresholds, which were in turn related to increased fussing and crying (42, 43).

In our sample, there was a high comorbidity of ERD with almost 50% of the sample fulfilling diagnostic criteria of more than one diagnosis. Accordingly, the scale "sum of symptoms" covers a large range of up to 35 clinically assessed symptoms of different ERD (**Table 2**). As an additional predictor, the QCFS sum score, reflecting the extent of mother-reported infant overall level of crying, sleeping, and feeding symptoms and co-regulation difficulties, independently was predictive for MPS. Thus, while our results highlight the importance of infant fussing/crying for PS, we also found that the overall level of symptoms in addition is relevant to the PSI. Our results imply that for a mother in our sample, the more infant symptoms the greater the levels of MPS, irrespective of the nature and quality of the symptoms or the behavioral area affected.

Our results support previous literature on the adverse effect of prolonged crying on parents' level of perceived burden and physiological reactions in no-risk and risk samples (40, 44, 45). While it is also likely that higher MPS, which renders parents less effective in soothing their child, contributes to more regulation problems, the literature points to negative effects of dysregulation on parents (4, 5). In addition, research demonstrated that duration of fussing and crying is a precursor of maternal depression (46). It is also likely, that both factors—MPS and infants' dysregulation—exist in a reciprocal relationship with each other, thereby contributing to the perpetuation of ERD (6). Drawing from our results, the overall number of infant symptoms and especially fussing and crying related to ERD contribute to this buildup, whereas other specific infant symptoms may be less important.

These results highlight the need to utilize multiple measures in order to estimate the association between regulatory symptoms and MPS. Behavioral diaries seem to capture important aspects of everyday life that are relevant to MPS. Self-report measures may add an important subjective factor to the clinically assessed symptoms. For treatment planning, our results suggest targeting mothers' experience of prolonged and inconsolable fussing/crying in sleeping disorders and comorbid ERD.

Infants' Age-Appropriate Physical Development

Mothers' rating of an age-adequate physical development of the child was the least important predictor in the final prediction model. While most of the mothers felt that their child was well-developed physically (94.08%, **Table 2**), it seems that having the impression of a "normal" development or not makes a difference to the extent of MPS. While interpreting this result, it is important to consider that infant age-appropriate developmental performance assessed with the ASQ-3 (e.g., gross-motor development), was unrelated to MPS. Thus, it seems that not the actual developmental problems but the mothers' perception thereof is what makes parenting in our sample more or less stressful. Asking mothers about their perception of infant

development may be a more valuable question in order to estimate their level of MPS related to ERD.

LIMITATIONS

While we assessed several risk factors, the use of cross-sectional assessed data in our study excludes causal data interpretation. In addition, although we included many empirically and theoretically derived variables, there are still more variables that have been shown to be related to ERD (e.g., perceived social support) and may predict MPS.

Our results' generalizability is restricted by the relatively homogeneous sample in terms of psychosocial and socio-demographic characteristics. This homogeneity led to close-to-zero variance, leading some variables to be excluded by the algorithm e.g., unemployment of one parent or both. Additionally, the exclusion criteria of this study likely limited the variance in relevant variables like organic and medical infant risk factors and maternal mental illness. While ML can handle different distributions, this has implications for generalizability. Thus, while we cross-validated all of our models within our dataset, it is likely that the final model does not generalize to unselected samples of mothers with infants presenting with ERD. For this reason, results of this study can only be generalized to similar clinical samples and will need future replication with more diverse samples and fathers.

We investigated a referred and self-referred clinical sample which restricts generalizability to mothers who do not seek help albeit experiencing the same problems with their child. However, we aimed to identify relevant predictors and correlates for MPS that may be utilized by clinicians to work more effectively and who likely see parents who seek help. Thus, our research question would not have been answered by investigating a representative sample.

Further limitations apply to some instruments used. PRFQ, MSES, and ASQ-3 are not validated in German. The Parent-Questionnaire covers some items which need further psychometric analysis (e.g., burden in pregnancy or infant development). The clinical interview utilized is not validated. However, infants' clinical characteristics in our study resemble other clinic and at-risk sample (3, 13), which speaks to the data's generalizability in this regard.

We used items in our dataset on a single-item level in order to maximize specificity and to make suggestions for future item selection. This strategy was further supported by the low reliability of some subscales (e.g., PRFQ-IC, SCL-psychoticism). However, readers should be cautious when interpreting our results not to infer an underlying construct from a single item.

FUTURE RESEARCH

With this study, we demonstrated that questions on the relative importance of multiple and interrelated factors in a complex field such as the one of infant mental health can be successfully investigated by utilizing ML. Based on this study, future longitudinal studies may utilize ML for the coverage of additional risk and protective factors (e.g., mental illness of both parents, social support) for PSI levels in both parents. Such investigations allow us to explore causal pathways that consider multiple infant and parent variables and their interactions within a family- and a developmentally sensitive perspective on the factors that contribute to PS in ERD. Future studies with naturalistic samples will lead to greater generalizability of the findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee Heidelberg University, Medical Faculty. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG and PS-P initiated this research and conceptualized the study. AG was responsible for data acquisition and data interpretation, and together with PS-P drafted the manuscript. PS-P and AG were responsible for data analysis. ST was involved in interpreting the results and drafting the manuscript. MC was involved in funding acquisition but passed away at the end of the year 2017, to the authors' great regret. All authors approved the final version of the 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.2021.663285/full#supplementary-material

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REVIEW
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Effects of Early Life Stress on the Developing Basolateral Amygdala-Prefrontal Cortex Circuit: The Emerging Role of Local Inhibition and Perineuronal Nets

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The links between early life stress (ELS) and the emergence of psychopathology such as increased anxiety and depression are now well established, although the specific neurobiological and developmental mechanisms that translate ELS into poor health outcomes are still unclear. The consequences of ELS are complex because they depend on the form and severity of early stress, duration, and age of exposure as well as co-occurrence with other forms of physical or psychological trauma. The long term effects of ELS on the corticolimbic circuit underlying emotional and social behavior are particularly salient because ELS occurs during critical developmental periods in the establishment of this circuit, its local balance of inhibition:excitation and its connections with other neuronal pathways. Using examples drawn from the human and rodent literature, we review some of the consequences of ELS on the development of the corticolimbic circuit and how it might impact fear regulation in a sex- and hemispheric-dependent manner in both humans and rodents. We explore the effects of ELS on local inhibitory neurons and the formation of perineuronal nets (PNNs) that terminate critical periods of plasticity and promote the formation of stable local networks. Overall, the bulk of ELS studies report transient and/or long lasting alterations in both glutamatergic circuits and local inhibitory interneurons (INs) and their associated PNNs. Since the activity of INs plays a key role in the maturation of cortical regions and the formation of local field potentials, alterations in these INs triggered by ELS might critically participate in the development of psychiatric disorders in adulthood, including impaired fear extinction and anxiety behavior.

Keywords: development, sex differences, corticolimbic circuit, perineuronal nets, amygdala, early life stress, fear, parvalbumin interneurons

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INTRODUCTION

Numerous human epidemiological and observational studies have strongly linked adverse early life experiences with consequences on cognitive and emotional health (Bremne and Vermetten, 2001; Pechtel and Pizzagalli, 2011; Gould et al., 2012). Emotional, sexual and physical abuse, as well as neglect contribute to chronic early-life stress (ELS) in children, increasing their vulnerability to

develop future psychiatric disorders, including anxiety, depression, and substance abuse (Weber et al., 2008; Green et al., 2010; Carr et al., 2013). It is estimated that 45% of childhood-onset mental health disorders and over 30% of later-onset of disorders are associated with early life adversity (Green et al., 2010; VanTieghem and Tottenham, 2017). The problem of childhood adversity is amplified because in humans as in non-human primates, there is a strong transgenerational transmission of infant maltreatment (Greene et al., 2020; Zeynel and Uzer, 2020). In macaques, stable transmission occurs mainly through the maternal line (Maestripieri, 2005; Morin et al., 2020) while in humans, both maternal and paternal early adversity can lead to adverse early experiences in their children. Adverse childhood experiences display marked sexual dimorphism in its occurrence, but also in the ensuing neurological and behavioral consequences which depend on the type, timing and duration of stress exposure (Fox et al., 2010; Gee and Casey, 2015; McLaughlin et al., 2019; White and Kaffman, 2019). For instance, girls are more likely to experience chronic sexual abuse, whereas boys are more likely to be exposed to physical violence and neglect (Maikovich-Fong and Jaffee, 2010; Gauthier-Duchesne et al., 2017; Coelho et al., 2018). Specific changes in gray matter volume (GMV) have been observed depending on the type of abuse encountered, with parental verbal abuse being associated with higher GMV in the adult auditory cortex and observations of decreased GMV in left and right visual cortex (V1) as well as cortical thinning of somatosensory cortex representing the genital areas in children exposed to sexual abuse (Teicher et al., 2016).

The heightened neuronal plasticity of the immature brain is usually advantageous and adaptive, allowing an individual to cope with and adapt to unfavorable conditions. However, this system may also become maladaptive in the face of early life adversity, dependent on genetic differences between individuals, and epigenetic changes of specific genes (McEwen, 2006; Buss et al., 2012; Franklin et al., 2012; Dunn et al., 2019). An alternative view is that ELS might selectively modify the way by which the brain processes information in a meaningful manner, leading to experience-dependent selective adaptations (Teicher et al., 2016). Structural and functional modifications would be geared toward ensuring increased survival and faster reaching of reproductive capabilities (Colich et al., 2020) in unfavorable conditions. This might come to the expense of accelerated aging since a recent report demonstrated that early life trauma predicted accelerated aging based on epigenetic clocks even though the trauma was not associated with age of menarche in this particular study (Hamlat et al., 2021). Childhood abuse, but not neglect, predicted faster epigenetic aging, emphasizing differences between the type of early adversity encountered. Similarly, a systematic review revealed that associations between early adversity and accelerated cortical thinning were region- and adversity specific, with threat-related adversity consistently associated with thinning in the ventromedial prefrontal cortex (vmPFC) (infralimbic, IL portion), and deprivation/neglect-type of adversity being associated with thinning in frontoparietal, default, and visual networks (Colich et al., 2020). Accelerated maturation of an adult-like type of corticolimbic connectivity has also been documented in individuals subjected to early life adversity (Gee et al., 2013a) even though a consistent association of ELS with amygdala-PFC connectivity was not detected in a recent large meta-analysis (Colich et al., 2020).

Current research efforts in humans and preclinical models are largely dedicated to understanding how early life perturbations begin to shape later physiological and behavioral responses in a sex-dependent manner (**Figure 1**) and to find specific windows of vulnerability amenable to successful interventions (Callaghan et al., 2014; Walker et al., 2017; Luby et al., 2020). The corticolimbic circuit including the amygdala (and in particular the basolateral amygdala, BLA), medial prefrontal cortex (mPFC), and ventral hippocampus (vHipp) is critically implicated in ELS-induced psychopathologies and more widely in the generation of symptoms of depressive and anxiety disorders (Shin and Liberzon, 2010; Burghy et al., 2012; Godsil et al., 2013; Gee and Casey, 2015; Yan et al., 2017). Dysfunction in the corticolimbic circuitry also predicts increased fear responses, stress hypersensitivity and negative behavioral outcomes.

Central to this circuit is the amygdala and in particular, the BLA nucleus and its efferent output projections. The amygdala is as heterogeneous functionally as it is anatomically; it plays a complex role in emotional and social behavior (LeDoux, 2000; Adolphs, 2010), stress response (Roozendaal et al., 2009), learning and memory, anxiety (Rauch et al., 2003), and conditioning to appetitive or aversive stimuli (Shabel and Janak, 2009). In humans, ELS enhances amygdala reactivity during the presentation of negative emotional stimuli and weakens amygdala-prefrontal cortex (PFC) resting-state functional MRI (rs-fMRI) connectivity (Tottenham et al., 2011; Burghy et al., 2012; Pagliaccio et al., 2015). While connectivity between corticolimbic regions is established mainly through reciprocal glutamatergic projections involving principal pyramidal neurons, local inhibitory interneurons (INs) in each of these structures are critical in regulating the activity and function of principal neurons (PN) and ultimately regulate behavior. In addition, GABAergic INs are important for the assembly of neuronal circuits during critical developmental periods and they have been strongly associated with the development of psychopathologies (Marín, 2016). Interestingly, a subpopulation of local inhibitory neurons expressing parvalbumin (PV) is ensheathed by perineuronal nets (PNNs), which are proteoglycan rich assemblies derived from the extracellular matrix that control the opening and closure of critical windows of plasticity in several brain regions (Hensch, 2003, 2005). Experimental dissolution of PNNs within the corticolimbic circuit has allowed to unravel a critical role of these molecular assemblies in the formation and retrieval of adult fear memory (Ehrlich et al., 2009; Gogolla et al., 2009). Emerging studies are now also documenting that ELS significantly alters PNNs and their role of stabilizing synaptic inputs onto developing inhibitory INs. It is therefore conceivable that preclinical research on the activity of INs and their associated PNN structures after ELS will greatly enhance our knowledge of how the corticolimbic circuit develops in unfavorable conditions. Furthermore, it might allow for the specific targeting of IN activity in the design of novel therapeutic strategies.

In this paper, we will first review developmental aspects of the corticolimbic circuit and how these can be altered

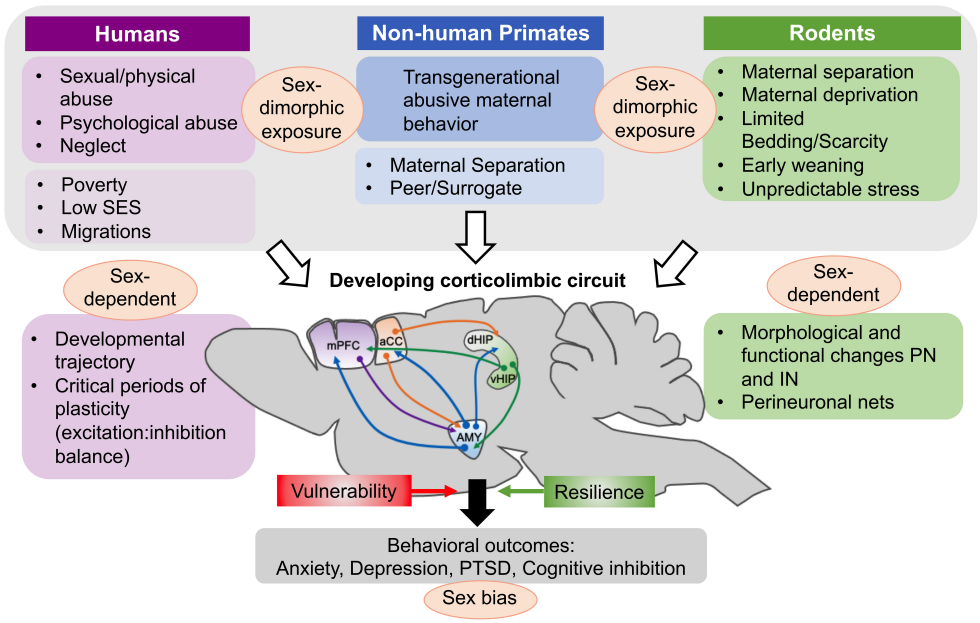

FIGURE 1 | Factors associated with early life adversity in humans and experimental procedures in non-human primate and rodent studies that have been used in studies of ELS and adversity. Several, but not all of these factors and procedures produce a sexually dimorphic exposure in the offspring. In humans, poverty, being raised in a low SES milieu or being part of migratory movements might be less directly associated with sex-differences in exposure. These modalities causing ELS have in common that they occur during a critical developmental period for the corticolimbic circuit, affecting the normal developmental trajectory as well as critical periods of plasticity characterized by changes in the balance of excitation and inhibition in amygdala (AMY), medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), and hippocampus (dorsal and ventral). At the cellular level, both morphological and functional changes in principal neurons (PN) and inhibitory interneurons (IN) have been documented after ELS and these vary as a function of the sex of the offspring. The important role of perineuronal nets (PNNs) in stabilizing synaptic inputs on interneurons and closing critical periods of plasticity is also modified by ELS. Based on anatomical, morphological and functional changes triggered by ELS, behavioral outcomes of ELS and childhood adversity are further modified by genetic risk factors and epigenetic changes that confer risk and resilience to the offspring. In all three species examined in this paper, behavioral consequences of early adversity are differentiated according to sex and specific behavioral categories. Figure adapted from Murthy and Gould (2020).

by ELS. We will then focus on some of the molecular and cellular changes observed in the amygdala and PFC that impact both principal neurons and inhibitory tone, and discuss how corticolimbic responses to ELS are impacted by sex and hemispheric differences.

AMYGDALA-PREFRONTAL CORTEX DEVELOPMENT IN HUMANS AND RODENTS

Amygdala Development

Studies across species have revealed that amygdala development is protracted, with many changes in morphology and function of the BLA occurring postnatally in both humans and rodents. Thus, environmental insults, such as ELS, during critical periods of amygdala neurodevelopment could lead to deviations in the normal maturational trajectory and subsequently affect the integrity of the amygdala. The human amygdala, including the basolateral complex, emerges early in embryonic life (Muller et al., 2006) and is well established at birth (Avino et al., 2018). A structural MRI study reported that the amygdala undergoes a 40% increase in volume between 8 and 18 years old in neurotypical individuals (Schumann et al., 2004; Uematsu et al., 2012). The dramatic increase in amygdala volume from youth to early adulthood is due to several factors, including an increase in the number of mature neurons, as well as increased synaptogenesis and dendritic growth during neuronal maturation. Remarkably, the increase in the number of mature neurons in the entire amygdala is primarily driven by increases in the number of BLA neurons (30%), and less so by neuron increases in other amygdala nuclei such as the LA, which only exhibit a 3% increase in mature neuronal counts throughout development (Avino et al., 2018). Although adult neurogenesis has not been demonstrated in the adult primate amygdala, a region located near the temporal lobe lateral ventricle and adjacent to the BLA called the paralaminar nucleus (PL), does, however, contain a large population of neurons exhibiting an immature phenotype in adolescence and adulthood (Sorrells et al., 2019). The PL in the primate amygdala is similar in location to the dense clusters of GABAergic cells found around the BLA in the rodent amygdala, called the intercalated nuclei. The observation of a protracted maturation of excitatory neurons in the human amygdala and the fact that the most substantial changes in the composition of the PL occur during adolescence suggests that these neurons might remain highly vulnerable to the effects of ELS. Indeed,

a recent study conducted in the macaque found that early life maternal separation modifies gene expression in the PL later in life (de Campo et al., 2017) and that neonatal hippocampal lesions accelerated the maturation of the PL neurons (Chareyron et al., 2016).

As in humans, the rat BLA forms in embryonic development (Berdel et al., 1997) since most neurons are generated between embryonic day (E) E14 and E17 (Bayer, 1980) and the total volume of the BLA increases until the third postnatal week (Rubinow and Juraska, 2009; Chareyron et al., 2011). Total neuron and glia numbers are unchanged from postnatal day (PND) 20 to PND35, but decrease by 13% between PND35-90 (Rubinow and Juraska, 2009). After PND35 and until adulthood, total BLA volume remains stable, but overall amygdala volume in female mice is generally smaller than that of males between PND30-90 (Koshibu et al., 2004). Principal BLA neurons undergo dramatic structural changes, particularly during the first postnatal month (Ryan et al., 2016). For instance, neuron soma size nearly doubles between PND7-28 and total dendritic length increases by three-fold until PND21 (Ryan et al., 2016). Dendritic arborization and spine density only reach maturity by PND28, which is comparable in age to early adolescence in humans (Quinn, 2005). Synaptogenesis in the BLA, as detected by presynaptic synaptophysin immunoreactivity, peaks at PND14 and stabilizes by PND30 (Morys et al., 1998). Increases in total synapse number during postnatal development may reflect the maturation of inputs to the BLA, particularly those coming from the mPFC which develop between PND13-21 (Bouwmeester et al., 2002b; Arruda-Carvalho et al., 2017).

Many of the morphological changes occurring in the rat BLA during postnatal development coincide with alterations in neuron physiology and synaptic plasticity. Passive membrane properties of principal BLA neurons, such as input resistance and membrane time constant, decrease with age and reach maturity at the end of the first postnatal month, in parallel with their structural development (Ehrlich et al., 2012). With regards to developmental changes in BLA synaptic plasticity, early studies have shown that high-frequency stimulation of LA inputs to the BLA results in modest long-term potentiation (LTP) on PND7-10, whereas after that and until PND19, long-term depression (LTD) is predominant (Thompson et al., 2008). The LTD state can be reverted to immature LTP via the application of $GABA_A$ receptor antagonists, suggesting that developmental changes in GABAergic transmission likely modulate synaptic plasticity in the neonatal BLA (Thompson et al., 2008). Indeed, many aspects of GABA transmission in the BLA only mature at the end of the first postnatal month (Ehrlich et al., 2013). However, in contrast to earlier findings, successful LTP in the BLA was demonstrated as early as PND20 in male and female rats without any pharmacological blockade (Bender et al., 2017). Around the end of the second postnatal week in the BLA (Ehrlich et al., 2013), there is a fundamental switch of GABAergic transmission from depolarizing to hyperpolarizing that has also been documented in the mPFC and hippocampus (Ganguly et al., 2001; Le Magueresse and Monyer, 2013). This early excitatory GABA function might thus facilitate weak LTP in the young BLA in the absence of $GABA_A$ receptor antagonists (Thompson et al., 2008).

Amygdala-Prefrontal Cortex Circuit Development

A rich body of human and rodent literature has identified robust structural and functional connections between the amygdala and PFC during the developmental period (Bouwmeester et al., 2002a,b; Kim and Whalen, 2009; Gee et al., 2013a,b). In humans, the developmental trajectory of amygdala-PFC circuitry is hierarchical in nature, because the amygdala displays early functionality relative to the PFC (Tottenham and Gabard-Durnam, 2017). Functional magnetic resonance imaging (fMRI) data have shown that the amygdala in children (ages 4–9) exhibits strong reactivity to emotional stimuli (i.e., fearful faces) before mature connections with the PFC are established (Gee et al., 2013b). Furthermore, functional connectivity in response to fear between the amygdala and PFC switches from positive in early childhood to negative at age 10, and then becomes progressively more negative into young adulthood (ages 18–22) (Gee et al., 2013b). The exact nature of these reciprocal connections remains unclear, although some studies have suggested that negative amygdala-PFC functional connectivity in adolescence and adulthood results from the development of active inhibitory PFC influence on the amygdala (Hariri et al., 2003; Kim et al., 2003; Hare et al., 2008). Thus, it is conceivable that early amygdala activity is able to instruct development of the PFC. As the PFC matures, top–down signaling increases with age and likely contributes to the observed valence switch in connectivity (Gee et al., 2013b; Tottenham and Gabard-Durnam, 2017).

In neonatal rats (PND10-12), the onset of fear learning relies on the postnatal maturation of anatomical connections between the BLA and mPFC (Raineki et al., 2010; Tallot et al., 2016), which is predominantly achieved in the second and third postnatal weeks (Bouwmeester et al., 2002b; Arruda-Carvalho et al., 2017). Specifically, BLA efferents to the IL and prelimbic (PL) regions of the mPFC emerge between PND7-9 and reach an adult-like innervation pattern in the mPFC by PND13. Most of the BLA-mPFC projections discovered in preweaning (PND18-20) rats are intra-hemispheric, but few inter-hemispheric connections also exist at this age (Verwer et al., 1996). The number of BLA to PFC projections continues to increase throughout adolescence and only stabilizes in adulthood (Cunningham et al., 2002). The descending mPFC to BLA projections mature later, between PND13–21 (Bouwmeester et al., 2002b) and remain relatively stable until they undergo pruning in late adolescence (PND45) (Cressman et al., 2010). Thus, as described previously in humans (Tottenham and Gabard-Durnam, 2017), there is a sequential type of maturation between the ascending and top–down components of this bidirectional circuitry that influences the development of behavioral responses to fear, fear extinction and fear memory.

Development of Amygdala-Related Fear Behaviors in Rodents

Most of our understanding of the corticolimbic fear circuitry has emerged from seminal studies using Pavlovian conditioning, an experimental paradigm that is frequently implemented to examine fear learning in both humans and rodents

(Fanselow and LeDoux, 1999; Maren, 2001; Carrere and Alexandre, 2015). Fear expression may be decreased through fear extinction, which is considered a different learning process (Krabbe et al., 2018) that occurs in three phases known as acquisition, consolidation and retrieval (Quirk and Mueller, 2008; Lee et al., 2015). Once the fear memory is consolidated, it may be reinstated by presenting the conditioning stimulus (CS) in the same context in which extinction occurred (Quirk and Mueller, 2008) or renewed by placing animals in a novel context (Marschner et al., 2008; Maren et al., 2013). Importantly, the renewal of context-dependent fear memories is developmentally regulated in rats, since extinguished fear responses can only be reinstated after the third postnatal week (Kim and Richardson, 2007). Before this time, fear experience is erased and there is no fear recall when rats are placed in a novel context (Kim and Richardson, 2007).

The protracted postnatal development of the corticolimbic circuit contributes to changes in the maturation of emotional behavior (Ryan et al., 2016; Sullivan and Opendak, 2020a). During the first week and a half of postnatal life, rat pups have limited hearing and vision and quickly learn to preferentially recognize maternal odors which promotes caregiver attachment (Sullivan, 2001; Tallot et al., 2016; Sullivan and Opendak, 2020b). Many studies using conditioning paradigms in neonatal rodents have therefore used olfactory cues as the CS (Jovanovic et al., 2013). Elegant studies have shown that during the first 2 weeks of postnatal life in rats, the mother has the ability to modify the emotional valence of stimuli (Moriceau and Sullivan, 2006; Santiago et al., 2017; Sullivan, 2017) even though pups can learn aversive responses after PND10. Before that time, PND8 rats are able to pair an odor and a foot shock (US), but the conditioned response is approach rather than avoidance to the shock (Sullivan et al., 2000). Once pups leave the nest around PND10, this same odor-shock conditioning can produce avoidance responses (Moriceau et al., 2006; Thompson et al., 2008), although when conditioning occurs in the presence of the mother, approach rather than avoidant responses to the CS are observed. When pups grow in the presence of an abusive mother, the beneficial buffering effect of the mother is lost and early avoidance responses to shock are observed. Other aspects of emotional responses such as freezing and fear-potentiated startle also mature along the development of the corticolimbic circuit. Conditioned freezing in rats emerges on PND16-18 (Hunt et al., 1998; Barnet and Hunt, 2006; Ryan et al., 2016) and reaches adult-like levels by PND23-27 (Jovanovic et al., 2013). Similarly, fear-potentiated startle to a tone is observed after weaning on PND23, but not earlier in the rat (Hunt et al., 1994).

Fear extinction outcomes and cellular mechanisms across development are also fundamentally different. In neonates (PND16), extinction training promotes the permanent erasure of fear memories (Kim and Richardson, 2007, 2008). By contrast, the same protocol in PND23 rats reinstates extinguished fear, as seen in adulthood (Kim and Richardson, 2007, 2008, 2010). This switch appears to be mediated by developmentally regulated changes in NMDAR activity in the brain (Langton et al., 2007), as their blockade only disrupts fear extinction on PND23, while PND16 extinction is unaffected. PNNs that surround primarily GABAergic neurons may also play a significant role in protecting fear memories from erasure at later developmental stages because enzymatic "dissolution" of PNNs in the BLA of adult rats reinstated the phenotype of erasure of fear memory that is normally only observed in young neonatal rats (Gogolla et al., 2009). Lastly, inactivation of the amygdala [BLA, lateral amygdala (LA), central amygdala (CeA)] disrupts extinction on PND17 but not on PND24 (Kim and Richardson, 2008), suggesting that extinction becomes more dependent on the mPFC once reciprocal connections between the BLA and mPFC are established (Kim and Richardson, 2008; Sotres-Bayon and Quirk, 2010; Jovanovic et al., 2013).

Summary

In both humans and rodents, development of the corticolimbic circuit is not achieved until the juvenile, peri-adolescent period, with ascending projections from the amygdala to the prefrontal cortex maturing earlier than top–down control of the amygdala by the PFC. The progressive change in connectivity between these two regions, follows important maturation processes in the amygdala with large increases in volume, synaptogenesis and dendritic arborization occurring up to the third week of life in rodents and 18 years in humans. The protracted and predominantly postnatal development of the corticolimbic circuit in human and rodents makes this circuit particularly sensitive to environmental stressors occurring during the neonatal and juvenile periods (Bouwmeester et al., 2002a,b; Gee et al., 2013a; Tottenham and Gabard-Durnam, 2017). As a consequence, the acquisition of fear can be accelerated in adverse conditions and in particular, negative, more "mature" connectivity between the amygdala and the PFC is observed in children raised in institutions compared to those raised in their biological families (Gee et al., 2013a). Similarly, the perception of an aversive situation is observed shortly after the first week in rodents, although it is usually buffered by the presence of the mother. In the case of an abusive mother, the buffering effect is lost and rat pups exhibit enhanced aversive learning. Conditioned freezing to an aversive shock is learned by the 2nd or 3rd week of life in rodents, although fear memory only appears after weaning in parallel with the maturation of top down projections from the PFC and amygdala that are responsible for fear extinction. Because of the sequential maturation of the ascending (fear acquisition) and descending (fear extinction) projections in both human and rodents and the sensitivity of the maturing amygdala to stress, it is understandable that exposure to early life stress and trauma has such severe consequences on the exaggerated processing of fear and sustained fear memory that ultimately leads to an anxiety phenotype.

IMPACT OF EARLY LIFE STRESS ON THE CORTICOLIMBIC CIRCUIT

Early adversity produces robust changes in the amygdala and amygdala-prefrontal pathway across species that associate with emotional difficulties throughout the life-span (Ellis et al., 2004; Burghy et al., 2012; Gee and Casey, 2015; Hanson et al., 2015).

Major changes are observed in the structure and excitability of the amygdala as well as in the functional connectivity between the amygdala and the PFC. These changes are long-lasting and might well represent the neural underpinnings of anxiety and mood disorders.

Structural Alterations in the Amygdala Following Early Life Stress

The amygdala is one of the few structures that generally increases in volume in response to chronic stress (Vyas et al., 2002; Mehta et al., 2009; Tottenham et al., 2010) and this might be related to the high density of glucocorticoid receptors present in the amygdala (Geuze et al., 2012). The amygdala is extremely sensitive to the effects of both acute (Mitra et al., 2005; Rodriguez Manzanares et al., 2005; Duvarci and Pare, 2014; Kim et al., 2014) and chronic stress exposure (Vyas et al., 2002, 2004, 2006; Tottenham et al., 2010; Rau et al., 2015). In humans, somewhat conflicting results on the effects of early life adversity on amygdala volume have been reported depending on the developmental timing of stress exposure, as well as the duration and intensity of the stress (Lupien et al., 2009; Fox et al., 2010; Gee and Casey, 2015). For instance, children reared in orphanages display significantly larger bilateral amygdala volumes (Mehta et al., 2009; Tottenham et al., 2010), while children who suffered from physical abuse or early neglect were found to have significantly smaller left amygdala volumes (Hanson et al., 2015). Similarly, children exposed to maternal depressive symptomology since birth (i.e., prolonged stress) exhibit increased amygdala volume, while hippocampal volume remains unaffected (Lupien et al., 2011). Interestingly, a recent study found that self-reported childhood neglect was associated with sex-specific and lateralized changes in the adolescent amygdala, with boys, but not girls, having larger right amygdala volumes (Roth et al., 2018). In adulthood, greater exposure to chronic stressors during childhood, measured using an index of cumulative risk exposure, is positively correlated with enlarged amygdala volumes (Evans et al., 2016).

Consistent with many human studies, the rodent BLA also undergoes hypertrophy in response to chronic stress. Juvenile chronic restraint stress (PND20-41) increases BLA pyramidal neuron dendritic length in male and female rats (Eiland et al., 2012). Similarly, chronic immobilization stress (CIS, 2 h/day for 10 days) in adult rats enhances dendrite arborization of pyramidal and stellate neurons in the BLA (Vyas et al., 2002). Interestingly, the same stress paradigm does not impact neuron morphology in the CeA (Vyas et al., 2003). Chronic stress also alters neuron morphology in the hippocampus by reducing spine density and arborization, however these changes are reversible, unlike those observed in the BLA (Vyas et al., 2003, 2004). Even after animals are given a 21-day stress-free period following CIS, the dendritic arbors in their BLA continue to increase in size, indicating that the amygdala may be 'sensitized' and thus more resistant to recovery following chronic stress (Vyas et al., 2004; Gee and Casey, 2015).

While the effects of chronic stress on the amygdala have been well documented in adults, less understood are the effects of chronic ELS on the structure of the neonatal and juvenile amygdala. Prenatal stress was shown to increase BLA volume, as well as neuron and glia density in male juvenile (PND25) rat offspring (Kraszpulski et al., 2006). Even though these structural differences appear to dissipate by adulthood, it is suggested that the accelerated BLA growth trajectory could impair developing reciprocal connections with the mPFC and enhance fear behaviors in the long-term (Kraszpulski et al., 2006). In our studies, we did not find significant volumetric changes in the preweaning amygdala after ELS, although there was a clear trend toward an increased volume (Guadagno et al., 2018a,b). ELS in the form of early weaning (on PND14) causes precocious myelination in the BLA of adolescent male mice (Ono et al., 2008). Collectively, these findings indicate that the consequences of ELS on the amygdala can be observed early as alterations in amygdala structural integrity in neonates, such as increased spine density that is observed exclusively in males (Guadagno et al., 2018a) occur in parallel with enhanced amygdala reactivity and neuron excitability (Guadagno et al., 2020).

Effects of Early Life Stress on Amygdala Reactivity and Excitability

Humans with a history of ELS typically have larger amygdala volumes that presumably contribute to heightened amygdala reactivity to emotional stimuli (Tottenham et al., 2010). Retrospective (Dannlowski et al., 2013; Van Harmelen et al., 2013) and recent prospective studies (Javanbakht et al., 2015; Evans et al., 2016) of adults exposed to early adversity have identified exaggerated amygdala reactivity to negative emotional cues. In line with these findings, children and adolescents (all right-handed, males and females) that were previously institutionalized display right-lateralized hyperreactive amygdala responses to fear faces (Gee et al., 2013a). Youths having experienced caregiver deprivation or emotional neglect similarly display enhanced amygdala activation during the analysis of detailed emotional faces, but only on the left side (Maheu et al., 2010). In this case, left as opposed to right-lateralized amygdala activity may be due to the type of task used that engaged more extensive and conscious emotional processing (Maheu et al., 2010). Interestingly, the earlier the onset of childhood maltreatment, the more severe the hyperreactive amygdala responses to emotional stimuli, which further emphasizes the importance of the timing of postnatal ELS exposure in predicting amygdala outcomes (McCrory et al., 2013). It is worth noting that adverse rearing conditions increase amygdala reactivity to fearful relative to neutral faces in children (Tottenham et al., 2011), a response normally only observed in naïve adults (Thomas et al., 2001), suggesting that ELS might enable precocious amygdala development.

Amygdala reactivity in rodents is similarly enhanced following exposure to chronic stress during early life and adolescence (Raineki et al., 2012; Malter Cohen et al., 2013; Rau et al., 2015). More specifically, amygdala neural activity, measured with c-Fos immunohistochemistry, is increased after exposure to acute forced swim in adolescent male and female rats subjected to ELS (Raineki et al., 2012). ELS in the form

of altered maternal care between PND2–21 also increases the expression of c-Fos protein in the BLA of juvenile and adolescent male mice (PND26–34) exposed to a threatening context (Malter Cohen et al., 2013). ELS-induced changes in the development of the amygdala might result from dysregulated HPA axis activity that is associated with ELS. For example, the emergence of amygdala fear reactivity is accelerated by increasing CORT levels systemically or by injecting CORT directly in the amygdala of male and female neonatal rats (Moriceau et al., 2006). Following adolescent social isolation, adult male rats show hyperexcitability of BLA pyramidal neurons (Rau et al., 2015). In summary, the few existing studies on the effects of ELS on the juvenile or adult amygdala emphasize amygdala hyperexcitability and increased emotional behavior. Increased synaptic plasticity found in the right BLA of juvenile male rats exposed previously to ELS (Guadagno et al., 2020) might constitute one of the mechanisms leading to amygdala hyperexcitability after ELS, seeing as chronic stress in adulthood increases LTP formation in the amygdala (Dalton et al., 2012; Suvrathan et al., 2014).

Functional Amygdala-mPFC Connectivity Is Altered After Early Adversity

Studies in both rodents (Yan et al., 2017; Johnson et al., 2018; Guadagno et al., 2018b; White et al., 2020) and humans (Burghy et al., 2012; Gee et al., 2013a; Peverill et al., 2019) have demonstrated that functional connectivity within the corticolimbic circuitry is extremely sensitive to ELS exposure. In humans, early life adversity has been shown to weaken resting state amygdala-PFC functional connectivity in childhood (Gee et al., 2013b), adolescence (Burghy et al., 2012) and adulthood, suggesting that ELS exposure programs lasting changes in the functional integrity of the corticolimbic circuit. Based on results found in 7–8 year-old children, it was argued that heightened amygdala reactivity found in previously institutionalized youths accelerates the development of a more "mature" and weaker amygdala-PFC connectivity as assessed by fMRI in response to an emotional task (Gee et al., 2013b). However, a more recent study using a larger sample population has failed to replicate the pattern of changes seen in the study by Gee et al. (2013b), Zhang et al. (2019) and challenged the view that changes in the patterns of amygdala-mPFC connectivity with ELS truly reflects accelerated maturation of the corticolimbic circuit. Regardless of the notion of accelerated maturation, reduced functional connectivity between the amygdala and other brain structures important for emotional processing could explain the behavioral outcomes observed in the long term. In particular, reduced functional connectivity between the amygdala and anterior cingulate cortex (ACC) is also observed in children with high levels of prior cumulative stress exposure (Pagliaccio et al., 2015) and between the right amygdala and vmPFC in maltreated females, but not males (Herringa et al., 2013). Reduced connectivity between the vmPFC and left hippocampus was seen in both sexes (Herringa et al., 2013), emphasizing the fact that at least in humans, sex-related ELS effects exhibit hemispheric specificity that is dependent of the regional circuit considered. The reduced functional connectivity between the amygdala and several subregions of the prefrontal cortex was also observed in a longitudinal study in infant to juvenile macaques raised by abusive mothers (Morin et al., 2020). This effect was particularly observed in maltreated females and was partially predicted by increased exposure to cortisol in infancy.

Neuroimaging studies in rodents exposed to ELS have revealed equally disrupted patterns of amygdala functional connectivity (Holschneider et al., 2016; Yan et al., 2017) that persist until adulthood (White et al., 2020). For instance, exposure to unpredictable varied stressors (PND14–25) in mice increased amygdala-PFC, amygdala-vHipp (Johnson et al., 2018) and vHipp-PFC connectivity in adult male, but not female offspring (White et al., 2020). In this study, tractography revealed a significant hemispheric effect on amygdala-vHipp connectivity, with higher connectivity changes in the left hemisphere. When earlier time points were examined, early adversity in preweaning rats reduced rs-fMRI connectivity between the BLA and mPFC (Guadagno et al., 2018b) and this was also documented in adolescent and adult male rats (Yan et al., 2017). This suggests that there might be significant differences between ELS models, animal models (rat vs. mouse) or specific time windows associated with differential sex effects of ELS. Importantly, a recent study used high resolution *ex vivo* diffusion tensor imaging to visualize bilateral BLA-mPFC projections in PND56 rats and compare naïve to ELS-exposed rats. The results of this study showed an increased number of tracts crossing the midline (Bolton et al., 2018), indicating that ELS might cause aberrant inter-hemispheric structural, as well as functional connectivity.

Early life stress affects amygdala-related emotional behaviors, as indicated earlier, most studies on early adversity in children report an increased vulnerability to developing anxiety and mood disorders and it is thought that molecular, cellular and circuit-based modifications in the development of the corticolimbic circuit are essential mechanisms that promote such vulnerability. Examples are numerous. For instance, children reared in orphanages with greater amygdala volumes display more internalizing behaviors and anxiety (Tottenham et al., 2010), which are risk factors for the development of future psychopathologies (Hofstra et al., 2002). Indeed, roughly half of the children tested in this study met diagnostic criteria for at least one psychiatric illness, of which ∼20% were anxiety disorders (Tottenham et al., 2010). Higher levels of self-reported childhood neglect in adolescent males associate with enhanced right amygdala activity and elevated anxiety symptoms (Roth et al., 2018). Adults with anxiety disorders and a history of ELS also display enhanced amygdala activity to threat cues (Bremner et al., 2003a,b, 2005). It is believed that atypically large amygdala volumes in ELS-exposed individuals might allow for greater processing of negatively valenced information and increased amygdala sensitivity to fearful cues (Tottenham et al., 2010; Gee et al., 2013a). In addition, the heightened amygdala reactivity observed in early development might actually reduce the functional connectivity between the amygdala and the PFC (Burghy et al., 2012; Gee et al., 2013b), leading to a phenotype of

higher trait anxiety and emotional reactivity (Burghy et al., 2012; VanTieghem and Tottenham, 2017). Of interest is the notion that ELS might modify the number of fiber tracts crossing the midline that target cortical regions, as well as modify their integrity (Bolton et al., 2018; Howell et al., 2019). This is important since previous reports have shown that the structural integrity of white matter tracts between the amygdala and the PFC is inversely correlated with anxiety symptoms in adult humans (Kim and Whalen, 2009; Burghy et al., 2012).

In rodents, exposure to chronic stress during the developmental period also has lasting effects on the male adolescent and adult amygdala, as evidenced by increased anxiety and conditioned fear responses in these animals (Stevenson et al., 2009; Eiland et al., 2012; Molet et al., 2014; Guadagno et al., 2018a). Adolescent male rats reared under suboptimal conditions display increased BLA neural activity and enhanced anxiety and depressive-like behaviors (Raineki et al., 2012). Importantly, when the amygdala is temporarily deactivated, depressive-like behaviors in ELS animals are reversed, suggesting a causal link between amygdala functioning after ELS and behavioral dysfunctions (Raineki et al., 2012). Similarly, enhanced BLA neural activity observed in juvenile and adolescent mice exposed to ELS increases fear responses to a threatening context (Malter Cohen et al., 2013). Hypertrophy of BLA neurons in male and female adolescent mice following chronic juvenile stress associates with enhanced anxiety-like behavior in the elevated plus maze (Eiland et al., 2012). In line with the human literature (Gee et al., 2013b), reduced rs-fMRI connectivity between the BLA and mPFC after ELS is linked to long-term decreases in social interactions and increased depressive behaviors in adult rats (Yan et al., 2017).

Summary

In both human and rodents, exposure to early adversity increases amygdala volume and reactivity, in particular to fearful faces in children. The earlier the onset of maltreatment, the higher the magnitude of amygdala hyperactivity. Precocious myelination and increased synaptic plasticity has been observed in the rodent amygdala after early adversity, with males being more susceptible in general to the effects of early stress. In humans, it has been suggested that adversity might also enable precocious amygdala development. An accelerated maturation of the amygdala together with aberrant inter-hemispheric structural connectivity might precipitate the characteristic reduced functional connectivity of the amygdala with other brain regions demonstrated consistently across species. Most models of ELS exposure in humans, non-human primates and rodents have demonstrated that it creates a phenotype of enhanced anxiety and depression as well as reduced social behavior that is likely to be mediated, at least in part, by morphological and functional changes in the corticolimbic circuitry. Variations in the magnitude of the effects, the type of behavior mostly impaired and the possibility for resilience might depend on the genetic and epigenetic makeup of the individual as well as the timing and nature of the early stressors (Tottenham and Sheridan, 2009; Gee and Casey, 2015; McCarthy et al., 2017).

SEX AND HEMISPHERIC DIFFERENCES IN CORTICOLIMBIC DEVELOPMENT AS MODULATING FACTORS IN THE EFFECTS OF EARLY LIFE STRESS

Sex differences in the development of the rodent corticolimbic circuit and its specific components has been recently elegantly reviewed (Premachandran et al., 2020) and sex effects have been outlined at the level of the amygdala, mPFC and hippocampus in terms of differences in volume, morphology, synaptic organization, cell proliferation, microglia, and GABAergic signaling. Morphological and functional changes over the course of development have been studied mostly in the dorsal hippocampus, reporting both transient, and long-term effects of sex on this structure. Interestingly, females undergo an earlier switch in $GABA_A$-mediated excitation to inhibition during the first postnatal week in rodents, occurring between PND4–7 compared to PND8–14 in males (Premachandran et al., 2020). This difference in the timing of inhibition onset might be critical to determine further developmental processes in this structure and to identify potential windows of sensitivity to external stimuli. Consequences of sex-differences in maturation of the hippocampus have been found in the context of behaviors that are mediated by the dorsal hippocampus such as cognitive strategies for spatial learning (Shansky, 2018; Grissom et al., 2019). In contrast, few studies have investigated sex differences in the development of the vHipp regions and results of sex-related anxiety studies early in development have been somewhat contradictory.

The rodent amygdala, especially the BLA, has been the subject of far fewer studies examining sex and hemispheric differences during development. Work focusing on the medial amygdala (MeA), which is the region of the amygdala containing the highest concentration of estrogen and androgen receptors, has suggested that this amygdala subregion matures faster than other subregions in both sexes (Chareyron et al., 2011). Volumes of the MeA reach adult-like values faster in females (PND5) than in males (PND21) males, although increased total structural volumes, dendritic volumes and neuron numbers were observed in prepubertal (PND26–29) male compared to female rats (Cooke et al., 2007), independent of amygdala side. They also found that MeA neurons in males had more frequent miniature excitatory postsynaptic currents (mEPSCs) and a greater number of excitatory synapses on dendritic spines compared to females, again only on the left side (Cooke and Woolley, 2005). Enhanced MeA neuron numbers in males persist until adulthood (PND60) (Morris et al., 2008). For the developing rat BLA, no sex differences or interactions with amygdala hemisphere were found for total volume or number of neurons on PND20, 35, or 90 (Rubinow et al., 2009; Guadagno et al., 2018b), although exposure to ELS increased spine density in the BLA of males, but not female PND10 and PND21 rats (Guadagno et al., 2018a). There was also no significant effect of sex on maturation of physiological properties of BLA pyramidal neurons in the first postnatal month (between PND7–28) (Ehrlich et al., 2012). Only in adulthood do some sex and hemispheric differences emerge in the BLA

under normal circumstances. For instance, male adult rats have more dendritic spines on pyramidal BLA neurons compared to females (Rubinow et al., 2009), and greater numbers of PV-positive cells in the left compared to the right BLA (Butler et al., 2018). Thus, effects of sex and laterality in the rat emerge earlier in the developing MeA than in the BLA, highlighting that these amygdala nuclei are fundamentally different, both anatomically and functionally (Sah et al., 2003). If there are little underlying sex or laterality differences during the normal BLA developmental trajectory, sex and hemispheric-dependent effects of ELS on the young rat BLA (Guadagno et al., 2020) could likely be a direct consequence of the stress exposure.

In the human amygdala, a structural MRI study found that between 1 month to 25 years of age, the total growth period for males was longer than that of females, and likely contributed to the observed larger male amygdala volume (Uematsu et al., 2012). However, growth of the amygdala is not linear because the dramatic gains in volume at 5 and 6 years of age become more moderate as youths enter early adolescence with the volumetric growth of the left amygdala peaking earlier than the right amygdala (Russell et al., 2021). There is some discrepancy about sex differences in amygdala growth, some reports showing that the female amygdala volume (including white and gray matter) peak in pre-adolescence, 18 months earlier than in males, regardless of hemisphere (Uematsu et al., 2012) while others found that amygdala GMV is increased in males compared to females (ages 8–15), only on the left side, and varies according to pubertal stage and circulating testosterone levels (Neufang et al., 2009). These findings suggest that gonadal steroid levels might play an important role in governing sexually dimorphic amygdala development. Hemispheric differences in total amygdala volume are seen in males during development, but the direction of this asymmetry is related to the age range and type of amygdala tissue imaged. For instance, in contrast to results reported by Neufang et al. (2009), right asymmetry of amygdala volume was observed in males, but not females, from infancy until adulthood (Uematsu et al., 2012) and it is believed that the right and left amygdala develop at different rates and may be involved in different emotional regulation processes (Wager et al., 2003; Uematsu et al., 2012; Wen et al., 2017). Fetal testosterone has been shown to positively correlate with GMV in the left, but not the right, amygdala of typically developing boys (aged 8–11 years old) (Lombardo et al., 2012), suggesting that early organizing effects of sex hormones prenatally may contribute to lateralized amygdala volume. Other environmental factors in early preterm infants like exposure to musicotherapy in the NICU have been found to improve white matter maturation and increase amygdala volume compared to preterm infants with standard of care (Sa de Almeida et al., 2020).

While maturation of the hippocampus and amygdala occurs relatively early in development, that of the mPFC is not complete until young adulthood in humans, non-human primates and rodents (Cunningham et al., 2002). In the rat, there appear to be waves of development/pruning of the mPFC, in particular in the PL with a peak of volume around PND14 and PND24 and a gradual reduction in volume by PND35 (Van Eden and Uylings, 1985) associated with neuronal loss, which continues until adulthood (Markham et al., 2007). The neuronal decline is more pronounced in females, which exhibit a sharp decline around puberty onset (PND35–45) while in males, the neuron loss continues more linearly between periadolescence and adulthood (Willing and Juraska, 2015). Hemispheric mPFC differences have been documented in the adult rat, in particular for dopaminergic innervation and the role of the mPFC in neuroendocrine stress responses (Sullivan and Gratton, 2002), however, these studies have not examined the onset of mPFC laterality during development. Basal extracellular Dopamine concentrations, but not 5HT or norepinephrine exhibited sex-related lateralized differences in the vmPFC of adolescent rats, suggesting that differential maturation of neurotransmitter innervation of mPFC could participate in lateralized mPFC function already in adolescence (Staiti et al., 2011). Exposure of neonatal rats to ELS in the form of early weaning and unpredictable adolescent stress showed that the mean excitatory latency of vmPFC neurons to *in vivo* stimulation of the amygdala was longer in ELS compared to control rats in the right compared to the left hemisphere (Ishikawa et al., 2015), supporting impaired connectivity that could be more pronounced in the right vs. left vmPFC.

A large number of studies have found that neuronal, functional and behavioral outcomes of ELS are sexually dimorphic (**Figure 1**) and recent reviews have addressed this topic (Loi et al., 2014; Walker et al., 2017; White and Kaffman, 2019; Bath, 2020). In several experimental models of ELS, adult female behavior appears to be significantly less impaired than male behavior, but this should not be interpreted to indicate that females are entirely resilient to early adversity. In fact, many human studies have shown that females exposed to childhood maltreatment are more sensitive (White and Kaffman, 2019) and likely to develop stress-related mental disorders, such as anxiety and depression, compared to males (McCarthy, 2016; McCarthy et al., 2017). Other reports have specified that sex differences in the behavioral consequences of early adversity strongly depend upon genetic susceptibility and the type and developmental timing of maltreatment (Walker et al., 2017; White and Kaffman, 2019). In addition to being caused by chromosomal, epigenetic and hormonal differences in neurodevelopmental events, sexually dimorphic outcomes can also be due to differences in maternal care toward males and females after exposure to ELS as outlined in a review by Bath (2020). In the case of the development of the corticolimbic system, it is still unclear whether ELS affects the normal sexually dimorphic maturation of corticolimbic brain regions at different time points of development in male and female individuals or whether ELS affects maturational processes differentially between males and females.

EARLY LIFE STRESS MODULATES LOCAL AMYGDALA CIRCUITS AND INHIBITORY INTERNEURONS

When considering studies on the mechanisms of fear learning, extinction and fear memory, the large majority of studies have mainly considered the role of principal excitatory projection neurons (PN) because they exhibit varied response properties

that are input-selective and they are highly interconnected within several brain networks. PNs constitute the large majority of cells in areas of the corticolimbic circuit, and in particular, in the BLA and PFC, and their critical role in emotional processing has been demonstrated. However, there is mounting evidence to suggest that their activity is highly orchestrated by their interactions with local inhibitory, GABAergic INs (Lucas and Clem, 2018). Inhibitory GABA INs are diverse in many aspects such as their pattern of peptidergic co-expression, their axonal targets, firing characteristics and dendritic morphology. They can actively shape network activity and this is particularly relevant during the developmental period when both PNs and INs mature at different rates.

Local Amygdala Circuits in Fear Regulation

During acquisition and consolidation of conditioned fear, multimodal sensory information from the thalamus and cortex converges onto projection and GABAergic neurons of the LA, but also directly to BLA neurons (Romanski and LeDoux, 1993; Ehrlich et al., 2009; Duvarci and Pare, 2014). Activated LA projections terminate on glutamatergic cells in the BLA (Ehrlich et al., 2009), which engage separate disinhibitory mechanisms involving PV-positive and somatostatin-positive INs (Wolff et al., 2014) to facilitate excitatory output to the CeA. During the conditioned stimulus (CS = auditory tone), PV-expressing cells are stimulated and inhibit somatostatin INs, which results in dendritic disinhibition of BLA PNs (Wolff et al., 2014). Conversely, presentation of the unconditioned stimulus (US = footshock) inhibits both PV and somatostatin INs, through the action of vasoactive intestinal peptide (VIP)-positive INs on both PV and somatostatin neurons. This process of sequential feedforward inhibition ultimately leads to the disinhibition of BLA PNs, activation of CeA output neurons and enhanced fear learning (Ehrlich et al., 2009; Duvarci et al., 2011; Tovote et al., 2015; Krabbe et al., 2019; **Figure 2**). In addition to processing and relaying sensory information to the CeA, the BLA encodes (Gale et al., 2004) and stores permanent fear memories (Fanselow and LeDoux, 1999; Maren, 2001; Repa et al., 2001; Gale et al., 2004), a process that is dependent upon connections with the PL mPFC and vHipp (Arruda-Carvalho and Clem, 2014). Disruption of BLA activity not only impairs fear acquisition and conditioned fear responses (Amano et al., 2011), but also fear extinction (Herry et al., 2006). It is now recognized that within the BLA, functionally distinct populations of neurons encode fear acquisition (fear neurons) and fear extinction (extinction neurons). As illustrated in **Figure 2**, these neurons are differentially connected with the mPFC and vHIPP, in particular, to regulate many aspects of fear processing (Herry et al., 2008). Taken together, these data demonstrate that acquisition, extinction and memory of fear require precisely coordinated interactions between PNs and several subtypes of local inhibitory INs. Stress-induced disruption in the establishment and maturation of any of these local circuits is likely to have lasting consequences on fear processing and the storage of fear memories. Understanding how these local amygdala inhibitory circuits are established and where critical elements of regulation lie will help clarify some of the mechanisms through which, early life adversity dysregulates emotional behavior.

Amygdala Inhibitory Interneurons

Approximately 80–85% of the neurons in the BLA are glutamatergic PNs with spiny dendrites (McDonald, 1982) and their activity is coordinated by interactions with local INs, which constitute the remaining 20% of BLA neurons. PNs densely innervate the dendritic spines of other surrounding PNs, as well as the peri-somatic and distal dendrites of spine-sparse GABAergic INs (Muller et al., 2006). Reciprocally, most of the glutamatergic PNs within the BLA are the post-synaptic targets of INs, which can significantly influence their activity (Spampanato et al., 2011). The INs in the BLA are characterized by their expression of calcium binding proteins (calbindin, calretinin, parvalbumin) and/or neuropeptides like somatostatin, CCK or VIP (Muller et al., 2006; Spampanato et al., 2011) and their pattern of innervation of PNs (Muller et al., 2007; Woodruff and Sah, 2007). For instance, PV-positive cells with basket-type morphology synapse on the peri-somatic region of PNs, including the soma, axon initial segment, and proximal dendrites, whereas those with 'chandelier' morphology form another class of axo-axonic synapses, innervating the largest portions of the axon initial segment (Gabbott et al., 2006). In addition, PV-INs that constitute half of the BLA INs form synapses with INs from their own or other groups (Spampanato et al., 2011). They are not only critical in generating theta-range oscillations (30–80 Hz) (Marín, 2012), but are also differentially recruited according to the specific stimuli associated with fear conditioning and inhibition (Courtin et al., 2014; Wolff et al., 2014; Krabbe et al., 2019). IN firing properties are incredibly diverse and quite different from those of PNs (Rainnie et al., 2006). For instance, PV-cells in adult rats have more positive resting membrane potentials (−60 mV) than PNs and their burst-firing pattern requires high energy while other IN subtypes display regular and fast-firing patterns (Rainnie et al., 2006). The high frequency of action potentials observed in PV-positive INs and their dense excitatory innervation (Gulyás et al., 1999) makes them more sensitive to oxidative stress because of the higher metabolic requirements (Morishita et al., 2015; Steullet et al., 2017). Their higher sensitivity to oxidative stress might also make them more vulnerable to other types of stress from the environment. This is illustrated in a recent study showing changes in PV staining intensity, but not PV neuron numbers in the amygdala after early maternal separation in adult male and female rats (Gildawie et al., 2020a; Soares et al., 2020). It is interesting to note that in this particular study, maternal separation did not change PV staining intensity or cell number in the mPFC or in subfields of the hippocampus of either males or females, suggesting that effects on PV-INs are region-specific. However, ELS increased 8-oxo-dG in PV neurons, a marker of DNA oxidation in PV cells of the BLA and PL mPFC, indicating that PV neurons in these regions might be more sensitive to oxidative stress after ELS exposure (Cabungcal et al., 2013). In the mPFC, maternal separation

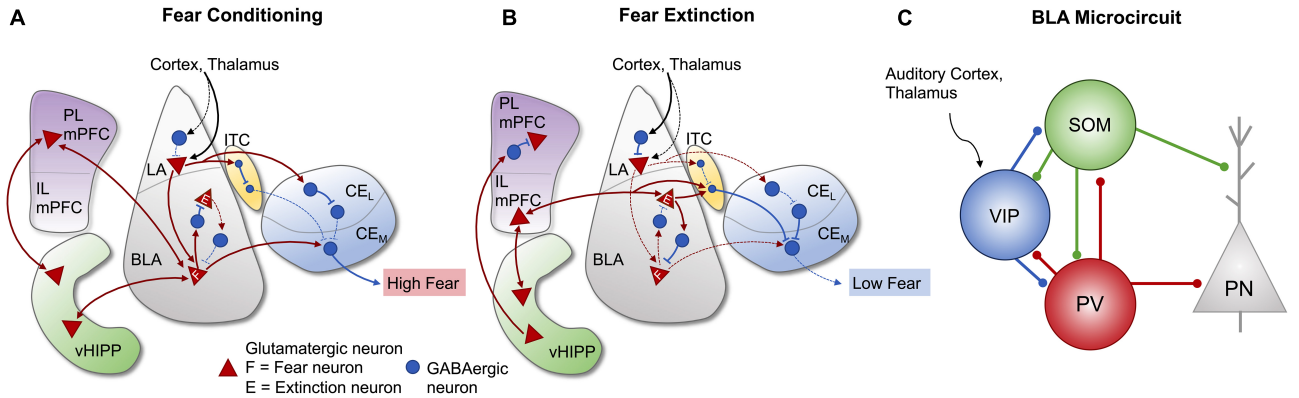

FIGURE 2 | Amygdala and corticolimbic pathways involved in fear conditioning and extinction. Strengthened and weakened connections are represented by the solid and dashed lines, respectively. **(A)** During fear conditioning, multimodal sensory information from the cortex and thalamus primarily converges onto lateral amygdala (LA) projection neurons and activates descending projections to fear neurons in the BLA, as well as inputs to GABAergic neurons in the intercalated cell masses (ITC). BLA fear neurons are also stimulated by projections from the ventral hippocampus (vHIPP) and prelimbic medial prefrontal cortex (PL mPFC). Output neurons in the medial division (Ce_M) of the CeA are disinhibited by GABAergic neurons in the ITC and lateral division of the central amygdala (Ce_L) and activated by BLA fear neurons, leading to high fear responses. **(B)** During fear extinction, incoming sensory information mostly converges onto LA inhibitory interneurons, leading to suppressed activity of LA projection neurons and decreased BLA fear neuron activity. Projections from the vHIPP inhibit PL mPFC activity and stimulate infralimbic mPFC activity (IL mPFC). The IL region activates glutamatergic extinction neurons in the BLA, which stimulate neurons in the ITC and inhibit BLA fear neurons, thus resulting in the inhibition of Ce_M output neurons and reduced fear responses. **(C)** Organized microcircuit of feedforward inhibition in the rodent BLA regulate the activity of principal neurons (PN) under associative fear learning. VIP interneurons receiving direct inputs from the auditory cortex and thalamus are strongly activated by unexpected aversive events (US: aversive shock) and they gate information flow to a large fraction of PNs by releasing them from the strong inhibition provided by SOM and PV interneurons. There is an extended reciprocal inhibitory connectivity between all three interneuron subtypes found in the BLA. Figure adapted from Ehrlich et al. (2009), Sotres-Bayon and Quirk (2010), Sotres-Bayon et al. (2012), Lee et al. (2013), Duvarci and Pare (2014), Arruda-Carvalho and Clem (2015), Krabbe et al. (2019), and Zimmermann et al. (2019).

led to a reduction in PV expression, as measured by Western blot, that appeared earlier in juvenile females than males (Holland et al., 2014).

In other regions of the corticolimbic circuit such as the hippocampus, distinct environmental exposures (i.e., either enrichment or fear conditioning) modulate the level of expression of PV in mice without necessarily modifying PV expressing cell numbers (Donato et al., 2013).

Development of Parvalbumin Inhibitory Interneurons

The importance of IN development and functionality within the corticolimbic circuits to regulate the inhibition:excitation ratio has been well described in previous reviews (Marín, 2012; Arruda-Carvalho and Clem, 2015; Lucas and Clem, 2018) and there is little doubt that early dysfunction in INs may underlie the emergence of several neurodevelopmental disorders (Marín, 2012) in addition to adult social dysfunction (Bicks et al., 2020) and anxiety. In rodents exposed to ELS, the rhythmic firing of neurons in the amygdala (theta and gamma oscillations range) is increased (Raineki et al., 2015), while theta and beta oscillations in the mPFC and ACC are reduced (Murthy et al., 2019; Murthy and Gould, 2020). Maturation of PV-neurons coincide with the early postnatal period when ELS has profound effects on the development of the corticolimbic circuitry. PV-positive neurons begin differentiating around PND10-14 in the mouse BLA (Dávila et al., 2008) and they reach a mature phenotype by PND30 (Berdel and Moryś, 2000), at the peak of inhibitory function in the mouse amygdala (Arruda-Carvalho et al., 2017). Exposure to ELS in the form of limited bedding (LB) conditions led to a transient increase in PV-cell density in the BLA of PND21 mice that was not observed in the mPFC (Manzano Nieves et al., 2020). By adulthood, the effect of LB on PV-cell density in the BLA was absent. This is consistent with another study in juvenile rats (PND28) under LB conditions that failed to document a significant effect of ELS on PV-positive cell density in the BLA in either males or females (Guadagno et al., 2020). The type of ELS paradigm might cause different region-specific changes in PV-INs in diverse periods of life. For instance, maternal separation in rats increased PV-cell density in the BLA only in adolescent males, but not females and this effect was not seen in the PL or IL regions of the mPFC (Gildawie et al., 2020b). Prenatal insults, such as maternal infection in late gestation has also been reported to induce a large increase in mPFC PV-positive neuron density in the offspring between PND14-28 (Boksa et al., 2016), a period that coincides with the expansion of threshold levels of inhibition important to trigger critical periods of cortical plasticity (Hensch, 2005). While the onset of critical periods is typically associated with the appearance of PV-positive neurons in several cortical areas (visual, auditory, mPFC), closure of these critical periods is more closely associated with maturation of PV neurons and their stable integration in functional circuit networks. An important component of the regulation of PV maturation and PV expression is the formation of PNNs that surround perisomatic synapses and proximal dendrites of particular subsets of neurons. The role of PNNs in stabilizing synaptic inputs preferentially on PV-positive IN

population (Freedom et al., 2014; Carceller et al., 2020) and in allowing for the binding of Otx2, a factor that is necessary for PV-cell maturation in the visual cortex (Bernard and Prochiantz, 2016) makes these molecular scaffolds important potential targets to ELS-induced cellular and network modifications (Kwok et al., 2011; Reichelt et al., 2019).

Development of Perineuronal Nets (PNNs) in Corticolimbic Regions

Perineuronal nets (PNNs) are lattice-like structures consisting of large molecular aggregates composed primarily of chondroitin sulfate proteoglycans (neurocan, brevican, aggrecan, and versican) and extracellular matrix components such as collagen, laminin and fibronectin as well as additional molecules (Tsien, 2013; Van't Spijker and Kwok, 2017). Components of PNNs are thought to be contributed by both neurons and glial cells (Carulli et al., 2006; Tanti et al., 2020) although their development is not yet fully understood. Evidence in animals suggest that PNNs may serve very different functions in specific brain regions, for example, loss of PNNs in the adult mouse visual cortex enhances LTP formation (de Vivo et al., 2013) whereas similar manipulations in the adult mouse LA reduces LTP (Gogolla et al., 2009). Furthermore, PNNs have been considered like "punch cards, in which the position and size of the holes (in the net) preserve the long-term location and strength of synapses" (Tsien, 2013). After a synapse is surrounded by the PNN, little synaptic reorganization occurs (McRae et al., 2007) and the synapse is stabilized into a mature state with limited AMPAR movement (Van't Spijker and Kwok, 2017; Bosiacki et al., 2019). The intensity of PNN staining is thought to be correlated with the maturation level of the PNN itself; as the holes tighten their grip on the perforating synapses throughout maturation and staining intensity increases (Sigal et al., 2019). PNNs protect fast-spiking PV-INs that are susceptible to oxidative stress (Cabungcal et al., 2013; Brenhouse and Schwarz, 2016) and provide a local buffer for cations that are close to the synapse. Although PNNs preferentially surround PV-INs, other neurons, including glutamatergic neurons also harbor PNNs, but likely to a smaller extent. Parvalbumin neurons surrounded by PNNs exhibit larger somata and higher level of PV expression compared to those not ensheated with PNNs (Enwright et al., 2016). High PV expression increases the calcium buffering potential of PV-positive cells and affects GABA release from these cells. In addition to their multiple cellular roles at the level of the synapse and in particular during developmental periods, recent data have suggested that the role of PNNs might extend into adulthood to modulate some of the cellular consequences of environmental changes. Indeed, maternal experience as a surrogate mother was found to increase the density of PNNs in the mouse primary somatosensory cortex and this increase was hemispheric dependent as a function of the specific cortical region considered (Lau et al., 2020). In this study, laterality of PNN expression was observed in naïve adult mice and disappeared once the mice became surrogate mothers, suggesting that the increased demand for tasks involving tactile stimulation in surrogate mothers might have triggered activity-dependent changes in PNNs that are potentially hemispheric differentiated. More generally, it is also tempting to entertain the idea that lateralization of PNN expression in the adult brain could contribute to functional hemispheric specialization by differentially regulating INs and even principal neurons.

It is not surprising that PNNs development and maturation are activity dependent (McRae et al., 2007), coinciding directly with the closure of critical periods of plasticity in several brain regions. In the visual cortex of the mouse, closure of critical plasticity occurs at the end of the first postnatal month (Hensch, 2003) and degradation of PNNs in the adult visual cortex results in atypical or "juvenile" ocular dominance plasticity (Pizzorusso et al., 2002) and enhanced LTP formation (de Vivo et al., 2013). Within the mouse BLA, PNNs emerge on PND16 and reach mature levels by PND28 (Gogolla et al., 2009) and in rats, PNN density gradually increased between juvenile and adult rats with no obvious sex-related differences in naïve rats (Gildawie et al., 2020a). In juveniles, the percentage of PNNs on total inhibitory GAD67-positive cells in the BLA approximated 40% (Guadagno et al., 2020). By adulthood, the majority of PNNs encapsulate PV-INs as well as a fraction of excitatory cells (Alpár et al., 2006; Morikawa et al., 2017). Interestingly, the appearance of PNNs in the mouse BLA coincides with a developmental switch in fear learning, such that conditioned fear memories are subject to context-dependent renewal following extinction training (Gogolla et al., 2009). The experimental degradation of PNNs on all BLA cell types in adulthood decreases LTP formation at LA inputs, reactivates critical period of plasticity and re-enables the permanent erasure of fear memories, a process normally only observed in neonatal mice. These results suggest that in the adult BLA, PNNs actively protect fear memories from being extinguished by facilitating synaptic strengthening (Gogolla et al., 2009). Degradation of PNNs in the hippocampus or mPFC also impairs the formation of conditioned fear memories in rats (Hylin et al., 2013).

In the mPFC, increased inhibition during adolescence is thought to contribute to a sensitive period for cortical plasticity that is associated with reaching adult-like levels of PV-positive cell density in this region (Baker et al., 2017). In contrast, the maturation of PNNs drastically increases between the juvenile period and adolescence in both IL and PL subregions of the mPFC. As a result, the number of PV-positive cells expressing PNNs reaches adult-like levels already by adolescence in the rat (Baker et al., 2017). The precise development of PNNs in the mPFC might follow a slightly different trajectory between male and female rats. In males, puberty did not affect the density of PNNs, but in females, the onset of puberty led to an abrupt reduction in the number of PNNs that persisted through mid-adolescence, before showing an increase toward adult levels at PND60 (Drzewiecki et al., 2020). This sex-dependent protracted PNN development in females in adolescence was observed in both IL and PL mPFC and might indicate a longer period of cortical plasticity in the mPFC. The rise in pubertal estrogens appears necessary for an increased inhibition within the ACC, although it is still unclear how estrogens modulate the expression of PNNs in these cortical regions (Piekarski et al., 2017).

Similar to rodents, PNNs develop in human PFC and hippocampus from as young as 2 months after birth with a

protracted course of postnatal maturation stabilizing between the ages of 8–12 years old (Mauney et al., 2013; Rogers et al., 2018). In the PFC, PNNs enwrap mainly PV-cells (Rogers et al., 2018), although they are expected to also enwrap excitatory cells in CA2 of the hippocampus and deep cerebellar nucleus as they do in rodents (Van't Spijker and Kwok, 2017; Bozzelli et al., 2018). Moreover, 31% of PV-positive cells in the human amygdala are ensheathed by PNNs (Pantazopoulos et al., 2006) leaving a large population of neurons that are covered to be characterized. Although human PNN studies are lacking, alterations in PNNs have been reported in post-mortem studies of various brain disorders. Deficits in PNNs are well documented in schizophrenia across many different brain regions, including the LA, superficial layers of the entorhinal cortex, HPC and layer III and V of the PFC (reviewed in Berretta et al., 2015). Furthermore, the intensity of labeling of both PNNs and PV + cells in the dorsolateral PFC (Enwright et al., 2016) is decreased which is thought to contribute to the dysfunction of microcircuits associated with this mental illness. In bipolar disorder, PNN reduction has been observed in entorhinal cortex layer II, but not in the PFC (Pantazopoulos et al., 2010; Mauney et al., 2013). Recently, Crapser et al. (2020) reported a specific decrease in PNNs in the subiculum of Alzheimer's disease patients. Despite these observations, little is currently known about the mechanisms underlying such disorder-specific changes nor about their functional consequences. It can be assumed, however, that changes in PNN/PV GABAergic neurotransmission may be at the root of altered gamma oscillations, an important rhythm in the BLA, which have been documented in multiple brain disorders (McNally and McCarley, 2016; Bozzelli et al., 2018; Blanco and Conant, 2020).

Early Life Stress Affects the Formation of PNNs

Because PNNs play an important role in regulating the activity of and synaptic inputs onto the neurons they surround, alterations in PNN structure and/or density by ELS may have strong consequences for neural functioning (Sorg et al., 2016; Reichelt et al., 2019). Exposure to ELS has been linked to aberrant PNN density and intensity in all regions included in the corticolimbic circuit (Riga et al., 2017; Page and Coutellier, 2018, 2019; Santiago et al., 2018), although the direction of effects is not always consistent between studies and brain regions. In rodents subjected to daily maternal separation (4 h/day) during the neonatal period, a reduction in PV cell density was observed in the mPFC of adolescent males, but not females (Soares et al., 2020) although PNN density was reduced in both sexes at PND40 (Gildawie et al., 2020a). The reduction in PNN density was observed mainly in the IL mPFC and not in the PL area. In contrast, the BLA of maternally separated rats exhibited increased PV and PNN density in adolescence, but only in males (Gildawie et al., 2020a). Despite the increased PV and PNN density in the BLA after maternal separation, the intensity of PV staining was reduced by ELS and correlated with an increase in 8-oxo dG staining, a marker of DNA oxidation (Soares et al., 2020). This suggests that maternal separation might affect the structure of PNNs, making them less efficient at protecting PV-neurons from oxidative stress. Indeed, prolonged periods of maternal separation paired with early weaning (MSEW) in mice altered the structural integrity of PNNs enwrapping PV-neurons in the vHipp of adult males (Murthy et al., 2019). In this region, MSEW reduced PV-cell density as well as the intensity of PV staining, but PNN density was unaltered and PNN intensity around PV-positive neurons in the subregion of the ventral dentate gyrus was actually increased, indicating that changes in PNN intensity might not always vary in parallel with changes in density. In other types of ELS, like the limited bedding conditions, PNN density was increased in the BLA of juvenile male rats and this effect was lateralized to the right amygdala exclusively (Guadagno et al., 2020; **Figure 3**). The increase in PNN density was not accompanied by a significant increase in PV-cell density in either sex, although the total proportion of PV-cells surrounded with PNN was increased in males (Guadagno et al., 2020), suggesting that the increase in PNN observed after the early stress of limited bedding targeted mostly PV-neurons.

To our knowledge, a single study has examined the possible consequences of ELS on PNNs in human post-mortem tissue. This recent paper reported an increase in PNNs in layers III, IV, V, and VI of the vmPFC of adult depressed suicides with a history of severe child abuse (Tanti et al., 2020). Along with the augmented density of PNNs, the authors observed an increase in intensity of the staining and coverage of enwrapped cells (**Figure 4**) that was associated with child abuse. Using *in situ* hybridization, the same study found that 65% of vmPFC PV-positive cells were surrounded by PNNs in psychiatrically healthy controls (Tanti et al., 2020) which is similar to the ratio calculated in rodent studies using IHC (Van't Spijker and Kwok, 2017). The ratio of neurons enwrapped by PNNs was disturbed in depressed suicides with a history of child abuse. This study suggests that ELS from sexual, emotional or physical abuse is associated with precocious maturation and increased recruitment of PNNs in the human vmPFC, which is consistent with the aforementioned stress acceleration hypothesis (Callaghan and Tottenham, 2016).

Together, the few studies that have examined the effects of ELS on the density and intensity of PNNs have raised important questions that will need to be addressed in the future. For instance, what are the mechanisms underlying regional differences in PV/PNN sensitivity to ELS? Are cell populations other than PV-positive inhibitory INs more likely to be enwrapped by PNNs in response to ELS? It is suggested that in normal BLA development, the increase in PNN density might be contributed to by an increase in PNN surrounding non-PV cells preferentially (Baker et al., 2017). Regardless of PNN density and intensity changes, is the molecular composition of the PNNs changing with ELS? Is there a PNN composition that is optimal for synaptic stabilization? PNNs surrounding certain cells (most likely pyramidal) appear more delicately/weakly stained in comparison to those surrounding PV-positive cells in the cortex; it will be important to determine whether the enwrapped cell type dictates PNN composition and whether ELS modifies this relationship between PNN intensity/composition and cell type.

Summary

Complex local intra-amygdalar circuits with multiple interactions between PNs and several classes of INs as well

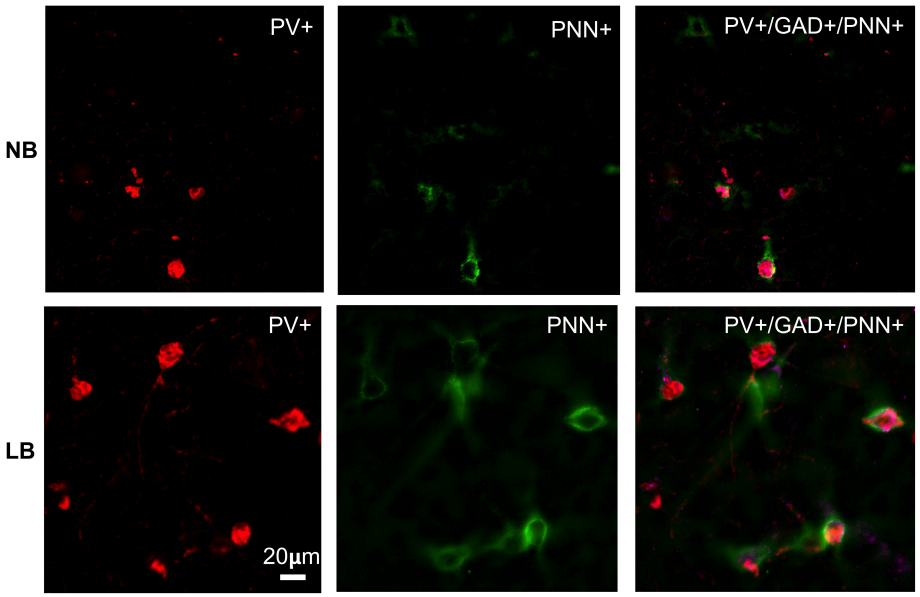

FIGURE 3 | Immunohistochemical detection of parvalbumin (PV) positive neurons in the juvenile (PND28) rat BLA that are surrounded by perineuronal nets (PNN) in offspring exposed to normal bedding (NB) or limited bedding (LB) conditions during the first 10 days of postnatal life. Note the higher density of cells co-expressing PV and PNN in the BLA of LB compared to control (NB) offspring. Images were taken on an Olympus BX63 microscope at 20x magnification. Scale bar is 20 µm.

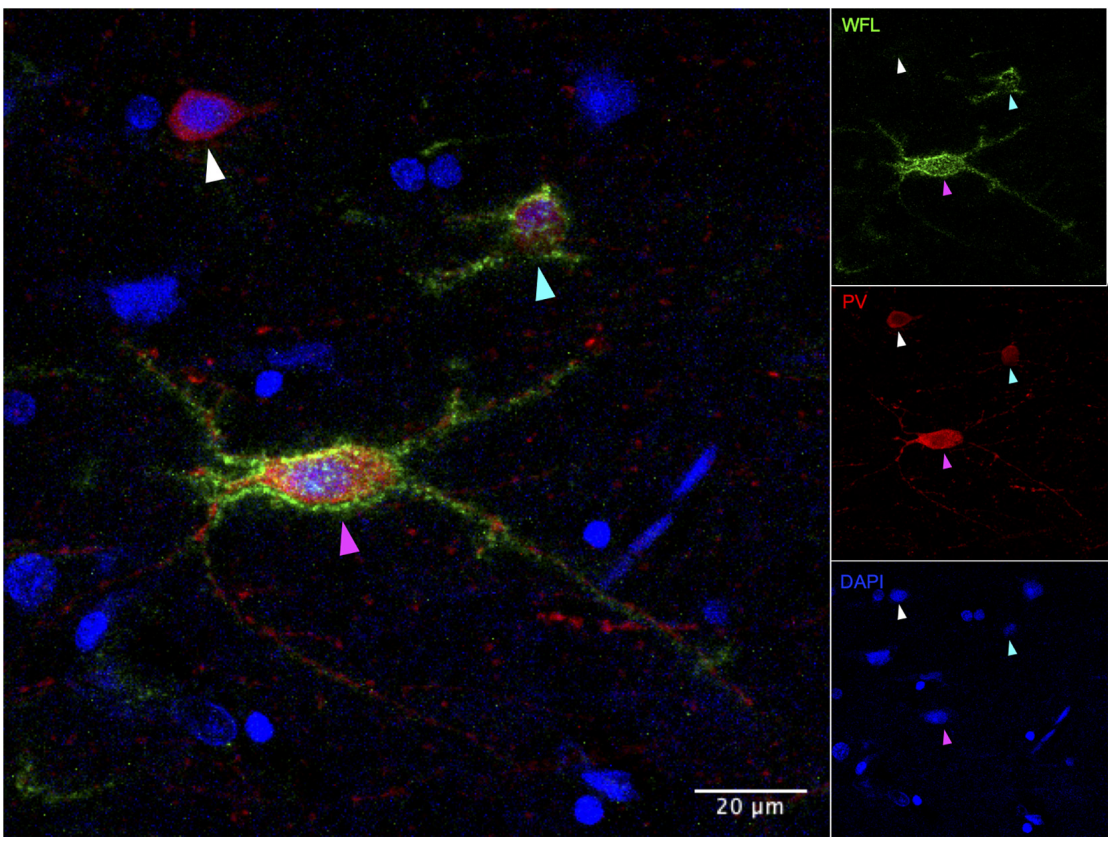

FIGURE 4 | Immunohistochemical detection of parvalbumin positive neurons in post-mortem human vmPFC that are surrounded or not by perineuronal nets. WFL, Westeria Floribunda Lectin; PV, Parvalbumin; DAPI, nuclear staining. White arrow: PV+/PNN– cell, Cyan arrow: PV+/PNN+ low intensity/coverage, Magenta arrow: PV+/PNN+ high intensity/coverage. Images were taken on an Olympus FV1200 laser scanning confocal microscope at 40x magnification.

within INs subtypes are fine-tuning the behavioral responses to aversive stimuli. In the amygdala and in particular in the BLA, 50% of the inhibitory INs are PV-cells that densely innervate excitatory neurons. Their PV expression and high firing activity is fully mature by PND30, at the peak of inhibitory activity in this structure. PV-neurons are surrounded by PNNs that protect them from oxidative stress and stabilize synaptic inputs. Interestingly, maturation of PNNs not only signals the termination of critical periods of plasticity within corticolimbic structures, but also allows for greater PV expression and GABAergic neurotransmission. It is perhaps not surprising to observe that ELS increases the expression of PNNs in the rodent BLA since PNN production is activity dependent. Increased PNN expression has also been documented after a history of child abuse in the human vmPFC. In contrast, in the rodent mPFC, ELS tends to reduce PNN expression and this might maintain PV-inhibitory neurons in a "labile" state longer than in the BLA, allowing for modifications and adaptations in neuronal input for longer time periods. Overall, multiple cellular processes participate in the organization and developmental regulation of corticolimbic activity, with plasticity in the formation of synaptic inputs terminating when the proper cellular scaffoldings are in place to insure stable and efficient inhibitory neurotransmission within local circuits. The fact that ELS significantly affects these specific cellular processes might constitute an additional target for treatment of aberrant fear memories and heightened states of anxiety in young and adult patients. In this respect, experiments showing that PNN dissolution in adult rodents could restore a developmental fear memory "phenotype" with the erasure of fear could constitute a promising mechanism to erase traumatic memories in PTSD and anxious patients. Similarly, the targeting of specific IN populations based on their phenotype and specific action on PNs might represent a useful addition to current psychopharmacological treatments.

CONCLUSION AND FUTURE DIRECTIONS

The dramatic and long-lasting consequences of childhood adversity and ELS have now been extensively documented in emotional, cognitive and social areas across species and sexes. The urgency to identify risk, but also resilience phenotypes after exposure to ELS has stimulated a large research effort with the overall goal to understand mechanisms, identify critical windows of susceptibility and plasticity, allowing for targeted and efficient interventions to curb the occurrence and severity of negative consequences. Functional connectivity and imaging studies from rodents to humans have consistently found region-specific and network-specific differences in the effects of ELS, in particular within the corticolimbic circuit. Accelerated development of the BLA and/or enhanced excitability in this structure have been proposed to drive the development of reciprocal BLA-PFC projections, modify cellular components of the network, and possibly reorganize critical periods of plasticity through effects on INs and PNNs. Pathways governing fear extinction are significantly altered after ELS, leading to the emergence of prolonged anxiety and other comorbid disorders. Here again, the formation of PNNs around INs is an important process that will set their connectivity and reduce their plasticity. Recovering a "labile" state of IN activity by altering specific components of the PNNs might represent an interesting future therapeutic prospect in the treatment of individuals with impaired fear extinction for instance.

The large number of studies performed on non-human primates and rodents have informed many of these processes, but also raised important questions that will need to be answered. For instance, how does ELS modify critical periods of plasticity (Reh et al., 2020) in the corticolimbic circuit in a risk and resilient phenotype? Are there cellular and molecular markers of vulnerability that reflect the variability in the outcomes of ELS? What are the molecular mechanisms through which ELS are affecting PNNs and local network activity in a region-specific manner? How does sex bias in preclinical experimental models reflect the observed sex-dependent cognitive and emotional consequences in humans? Future experiments should focus on elucidating potential mechanisms to explain sex differences in outcomes, investigating the role of early organizational effects of gonadal steroids and/or sex chromosomes on morphology, inhibitory tone and functional connectivity, identifying the primary IN subtypes that drive ELS effects and identifying ways to specifically target these, maybe through indirect effects on PNN integrity. Hopefully, answers to these questions will not only further our understanding of molecular and cellular processes underlying fear behavior, but also inform clinical and psychosocial teams to find the best time and modality of intervention to alleviate some of the dramatic consequences of early childhood adversity and trauma. We collectively owe this to our children.

AUTHOR CONTRIBUTIONS

AG and C-DW wrote the first draft of the manuscript, and edited and reviewed the final version. CB and NM wrote sections of the manuscript, and edited and reviewed the final version. All authors contributed to the article and approved the submitted version.

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