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Linking Maternal Disrupted Interaction and Infant Limbic Volumes: The Role of Infant Cortisol Output

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Abstract

Despite a large animal literature documenting the role of low maternal nurturance and elevated glucocorticoid production on offspring limbic development, these pathways have not yet been assessed during human infancy. Informed by animal models, the present study examined whether 1) maternal disrupted interaction is related to infant cortisol levels, 2) infant cortisol levels are

associated with infant limbic volumes, and 3) infant cortisol levels mediate associations between maternal disrupted interaction and infant limbic volumes. Participants included 57 mother-infant dyads. Infant saliva was measured at one time point before and two time points after the Still-Face Paradigm (SFP) at age 4 months. Five aspects of maternal disrupted interaction were coded during the SFP reunion episode. Between 4 and 25 months (*M* age = 11.74 months, *SD* = 6.12), under natural sleep, infants completed an MRI. Amygdala and hippocampal volumes were calculated via automated segmentation. Results indicated that 1) maternal disrupted interaction, and specifically disoriented interaction, with the infant was associated with higher infant salivary cortisol (AUCg) levels during the SFP, 2) higher infant AUCg was related to enlarged bilateral amygdala and hippocampal volumes, and 3) infant AUCg mediated the relation between maternal disrupted interaction and infant amygdala and hippocampal volumes. Findings are consistent with controlled animal studies and provide evidence of a link between increased cortisol levels and enlarged limbic volumes in human infants. Results further suggest that established interventions to decrease maternal disrupted interaction could impact both infant cortisol levels and infant limbic volumes.

Keywords:
Infancy; cortisol; maternal behavior; amygdala volume; hippocampal volume

Early life stress has been associated with elevated risk for depression, suicide attempts, anxiety disorders, and substance abuse (Teicher & Samson, 2013). Yet, the potential beginnings of these trajectories in infancy, a period of particularly rapid brain development (Gilmore et al., 2012), remain largely unstudied. Animal research has shown that stress response networks in early infancy are highly sensitive to adversity. Specifically, low maternal nurturance (Turecki & Meaney, 2016) or maternal unpredictability (Drury et al., 2016) is associated with increased

stress responses and altered amygdala volume and activation in rodent offspring. The current study in human infants assessed how disrupted maternal behavior is related to infant cortisol levels in response to stress in early infancy and, in turn, how infant cortisol levels are related to amygdala and hippocampal volumes during the first two years of life.

Animal Research on Neurobiological Effects of Stress in Infancy

Controlled studies with rodents show that manipulating the type and timing of early stressors leads to persistent alterations in amygdala development and function (Drury et al., 2016; Turecki & Meaney, 2016). The amygdala may be particularly vulnerable to the effects of early stressors due to high glucocorticoid receptor density (Peiffer et al., 1991) and to a postnatal developmental trajectory characterized by rapid initial growth and gradual pruning (Gilmore et al., 2012). Both environmental stressors and direct corticosteroid administration stimulate dendritic arborization and formation of new spines in the amygdala, increasing volume (Vyas et al., 2004; Vyas et al, 2006).

The hippocampus is also highly sensitive to stress. Early life stress reduces the number of hippocampal neurons, inhibits neurogenesis, and leads to abnormalities in synaptic pruning (e.g., Chen et al., 2012; Maras & Baram, 2012). Chronic early environmental stress, corticotrophin-releasing hormone (CRH) manipulation, and corticosteroid administration have been associated with reduced hippocampal volume and function, which are reversible when the stressor is removed (Chen et al., 2012; Maras & Baram, 2012).

Low Maternal Nurturance as Stress in Controlled Animal Research

The environmental stressors linked to increased activity of the hypothalamic-pituitary-adrenal (HPA) axis in rodent studies have predominately involved low maternal nurturance or unpredictability, indexed by variations in responsiveness of maternal care (Drury et al., 2016;

Turecki & Meaney, 2016). Low maternal responsiveness is associated with a host of alterations in the development of rodent pups, including changes in HPA axis output, circuitry of the amygdala and hippocampus, and behavioral functioning that persist into adulthood (e.g., Champagne et al., 2008; Drury et al., 2016; Turecki & Meaney, 2016). For example, Champagne and colleagues (2008) found that high corticosterone administration was associated with greater long-term potentiation in the hippocampus for pups exposed to lower maternal licking and grooming, potentially reflecting enhanced hippocampal plasticity under high-stress conditions. Similar findings of disrupted maternal care leading to offspring HPA-axis dysregulation have been demonstrated among non-human primates (e.g., McCormack et al., 2022; Sanchez et al., 2015).

Parent-Infant Interaction and Limbic Brain Volumes in Infancy

In human developmental research, few studies have assessed aspects of parenting in infancy in relation to brain volumes in infancy (Ilyka et al., 2021). Sethna et al. (2017), in a sample of 38 infants, found that maternal low positivity (positive communication and engagement) was associated with decreased total brain volumes among 4-month-olds, whereas lower maternal sensitivity, defined as timely, consistent responsive behavior, was associated with smaller subcortical grey matter volume (putamen, thalamus, globus pallidus, caudate). In addition, in a sample of 17 infants, Rifkin-Graboi et al. (2015) reported that lower maternal sensitivity was related to larger hippocampal volume, but not amygdala volume, among 6-month-olds.

Parent-Infant Interaction and Limbic Brain Volumes in Childhood and Adulthood

A larger set of studies has assessed parent-infant interaction in relation to limbic brain volumes in childhood or adulthood, using two observational measures: a) parental sensitivity

(responsiveness, positivity) and b) security of infant attachment behavior. Lower sensitivity and less secure attachment in infancy have predicted larger amygdala and hippocampal volumes at later ages (e.g., Bernier et al., 2019; Cortes Hidalgo et al., 2019; Khoury et al., 2019; Lee et al., 2019; Lyons-Ruth et al., 2016; Rao et al., 2010). In contrast, lower maternal nurturance or sensitivity assessed nearer to school age has shown inconsistent effects on hippocampal volume in childhood (Kok et al., 2015; Luby et al., 2012).

The *increased* limbic volumes generally seen in relation to lower maternal nurturance in infancy contrast with the *decreased* child and adult limbic volumes often related to childhood exposure to abuse (McLaughlin et al., 2019; Teicher et al., 2016). However, in a meta-analysis on maltreatment and hippocampal volumes, Reim et al. (2015) found evidence for reduced hippocampal volume among adults, but not among children. Thus, both developmental timing (infancy/childhood versus adulthood) and type of stressor (low maternal nurturance/maternal unpredictability versus threat of harm) may influence how adversity affects limbic development.

Parent-Infant Interaction and HPA Activity in Infancy

A large body of work indicates that maternal sensitive and responsive behavior in infancy is linked to more optimal patterns of infant cortisol regulation, particularly faster recovery to a mild stressor, whereas less sensitive caregiving is linked to more prolonged activation of the HPA axis (e.g., Atkinson et al., 2016, for review). Importantly, a wide range of insensitive caregiving is coded at the negative end of the spectrum, varying from harsh/intrusive behavior to pronounced lack of engagement.

Of most relevance to the current study, a large literature has examined infant behavioral and cortisol responses to the Still-Face Paradigm, the most widely used mild stressor for infants in the first six months of life (SFP; Tronick et al., 1978). In this procedure, the mother disrupts

normal interaction with her infant by displaying a “still face”. Although the still-face period reliably elicits infant negative affect (Mesman et al., 2009), infant cortisol responses over the course of the SFP do not typically follow a pattern of elevated cortisol reactivity followed by recovery. Instead, infant cortisol responses to the SFP vary in relation to family risk variables, with lower-risk infants showing decreasing patterns of cortisol release from baseline to recovery, whereas higher-risk infants exhibit sustained higher cortisol levels across the procedure (Crockett et al., 2013; Erickson et al., 2019; Khoury et al., 2021; Martinez-Torteya et al., 2014).

Cortisol Output in Infancy and Limbic Volumes

Among human infants, the theoretically important relation between cortisol output and limbic volumes has not yet been explored. Two studies of school-aged children reported that higher cortisol levels in response to a frustration-based laboratory stressor were associated with smaller amygdala volumes (Fowler et al., 2021; Pagliaccio et al., 2014). However, limbic volumes were assessed among older children rather than infants, and cortisol was assessed in relation to non-maternal stressors rather than in relation to aspects of maternal care.

The Present Study

The current study examined whether aspects of maternal disrupted caregiving in early infancy were associated with infant cortisol output and altered limbic volumes. Aims of the study were to 1) evaluate the association between aspects of disrupted maternal interaction and infant cortisol levels during a laboratory stressor at 4 months of age; 2) examine the relation between infant cortisol levels and infant amygdala and hippocampal volumes during the first two years of life; and 3) assess the extent to which infant cortisol mediated any relation between maternal disrupted interaction and infant amygdala and hippocampal volumes. Given the rapid development of the amygdala and hippocampus over the first two years of life (Gilmore et al.,

2012), moderation by age at MRI assessment was evaluated in all models related to limbic volumes.

Methods

Participants

Mother-infant dyads (n=57) were drawn from a larger cohort of 181 families enrolled in the Mother-Infant Neurobiological Development (MIND) Study. Mothers were recruited into the MIND Study through prenatal classes, obstetric and pediatric clinics, community flyers, and local birth records. Participants were recruited such that approximately half (57.1%) of the mothers had experienced one or more forms of childhood maltreatment (physical, sexual, emotional abuse; witnessed domestic violence; physical, emotional neglect). Exclusion criteria were: a) English not a primary language spoken at home, b) maternal age over 44 years at time of infant birth, c) infant born before 36 weeks gestation and/or weighing less than 2500g at birth, and d) infant congenital developmental disorder or birth defect.

The larger study included behavioral assessments in early infancy (4 months) and later infancy (15 months). Upon study entry, the first half of participating families were offered infant MRI scans after the later infancy behavioral assessment, and the second half of participants were offered scans after the early infancy behavioral assessment (all MRIs occurred prior to 25 months). Thus, all MRIs occurred after behavioral assessments. Infants who completed the MRI did not differ from infants who did not on demographic characteristics (infant sex, gestational age, minority status, family income, maternal education, p_{range} = .45-.77) or on maternal childhood maltreatment severity [$t(178) = -1.337$, $p = .18$]. See Supplement for more detail regarding reasons for unsuccessful scans.

Ethical Considerations

The study was approved by the Institutional Review Board [Partners Healthcare IRB Protocol #: 2014P002522]. All mothers gave informed consent for participation prior to the initiation of any study activities.

Measures

Still-Face Paradigm (SFP). Mothers and infants participated in the SFP (Tronick et al., 1978) when the infants were aged 4 months (*M* age = 4.54, *SD* = 0.85, range = 3.16 - 7.36). The SFP was administered in a comfortable area of the home to minimize the effects of travel on cortisol values. During the SFP, the mother interacted with her infant for 3 minutes (Play Period), then displayed a neutral face and did not interact with her infant for 2 minutes (Still-Face Period), then engaged in a final period of interaction for 5 minutes (Reunion Period). The SFP has been validated in meta-analyses as a mild to moderate behavioral stressor for the infant, resulting in reduced positive and increased negative affect (Mesman et al., 2009). A smaller literature on cortisol response suggests that elevated cortisol after the onset of the two-minute still-face stressor period occurs in approximately one-quarter of infants (Martinez-Torteya et al., 2015), particularly those at greater psychosocial risk. For example, sustained elevated infant cortisol levels are related to more severe maternal childhood maltreatment (Khoury et al., 2021) and more maladaptive maternal interaction (Crockett et al., 2013; Erickson et al., 2019; Martinez-Torteya et al., 2014).

Infant cortisol. Infant saliva samples were collected at three time points during the SFP: prior to initiation of the SFP (baseline), 20 min after the still-face period ended (+20 min), and 40 min after the still-face period ended (+40 min). The SFP was conducted in the afternoon (M=14:00h; *SD*=1h 38min) to avoid effects of expected diurnal changes in cortisol levels that typically occur over the morning. Infants did not eat or drink anything 30 min before the first

saliva sample was taken to avoid saliva contamination. Sorbette swabs (Salimetrics, State College, PA) were used to collect the saliva samples, which were stored frozen prior to processing. To collect saliva, swabs were centrifuged at 4 C for 15 min and 3000 rpm. Saliva samples were sent to the Kirschbaum laboratory at the Technical University of Dresden for assay. Samples were stored at -20 degrees C until analysis. After thawing, salivettes were centrifuged at 3,000 rpm for 5 minutes. Salivary concentrations were measured using commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany). Samples were assayed in duplicate and averaged values were used in analyses. Average inter- and intra-assay variability values were below 10%. Area under the curve with respect to ground (AUCg) and area under the curve with respect to increase (AUCi) were computed to represent total cortisol output and change in cortisol, respectively (Pruessner et al., 2003). AUCg represents total cortisol produced over the period from baseline through 40 minutes post-SFP (i.e., baseline cortisol levels influence AUCg values). AUCi represents change in cortisol from baseline to 40 minutes post-SFP (i.e., baseline levels do not influence AUCi values).

Maternal disrupted interaction. Maternal behaviors were video-recorded during the 5-minute Reunion Period of the SFP. Maternal behavior was coded from video recordings using the Atypical Maternal Behavior Instrument for Assessment and Classification (AMBIANCE; Lyons-Ruth et al., 1999). Coders first counted instances of relevant behaviors and then rated five dimensions of disrupted maternal interaction on 7-point scales, with higher scores indicating greater disrupted interaction: 1) *Affective communication errors* (incongruent affective signals to the infant or inappropriate or inadequate responses to the infant's cues); 2) *Role/boundary confusion* (soliciting the infant's attention or affection to the self in ways that override or ignore

the infant's signals); 3) *Disorientation* (disoriented, frenetic, or deferential behavior toward the infant, often characterized by odd affect, false affect, affect unrelated to the interaction, or affective disconnection); 4) *Negative-intrusive interaction* (harsh or critical verbal communication and/or physical behavior, such as mocking, teasing, or poking the infant); and 5) *Withdrawal* (creating physical or emotional distance from the infant, such as leaning away from the infant, averting gaze, sitting silently). After rating these five subscales, coders assigned an overall 7-point rating for Level of Disrupted Interaction. Interrater reliability between two coders on 30 randomly selected videos was strong on all scales: Overall Level ICC = .79; Disorientation ICC = .93, Withdrawal ICC = .80, Negative-intrusive interaction ICC = .88, Affective communication errors ICC = .94, Role confusion ICC = .90. AMBIANCE ratings of overall disrupted interaction have been shown to be stable over periods from 6 months to 7 years (Madigan et al., 2006) and have been validated in relation to a number of negative child outcomes from infancy to adolescence (Lyons-Ruth et al., 2013; Madigan et al., 2006; Yarger et al., 2020).

Sociodemographic interview. Sociodemographic factors were coded from parental interviews, including infant age, infant sex assigned at birth, infant minority race/ethnicity (details in Table 1), gestational age, annual family household income, maternal education, and mother living with partner.

Imaging Data Acquisition and Processing. All infant MRIs were performed on a 3.0 T Siemens Skyra scanner with a 64-channel head coil at Boston Children's Hospital. Infants were scanned during natural sleep, and no sedation was used. The T_1- weighted acquisition used an advanced version of the Magnetization Prepared Rapid Acquisition Gradient Echo (MPRAGE) sequence, where fast, low-resolution volumetric navigators were played each repetition period

and were used for prospective motion correction (Tisdall et al., 2012). The specific imaging parameters of the MPRAGE acquisition included: voxel size = 1 x 1 x 1 mm^3, repetition time (TR) = 2500–2540 ms, echo time (TE) = 1.65-2.37 ms, inversion time (TI) = 1450 – 1470 ms, field of view (FOV) = 192 x 192 mm^2 and between 144-173 slices, enough to cover the entire brain of the infant. After performing visual quality control using Freeview software (surfer.nmr.mgh.harvard.edu), the T_1-weighted volumes were manually aligned along the AC-PC plane and underwent N4 bias correction, field of view normalization (Ou et al., 2018), and multi-atlas skull stripping (Doshi et al., 2013). These steps were followed by automatic segmentation into cortical and subcortical regions, as well as tissue types classification using a multi-atlas-to-subject registration and fusion (Doshi et al., 2016) that has been extensively validated and adapted to infant brain MRIs (Ou et al., 2017). Quality control of the segmentations was visually performed using the FslView software. These segmentations enabled the extraction of right and left hemisphere amygdala and hippocampal volumes. Left and right hemispheric volumes were aggregated to derive total amygdala and hippocampal volumes for the current analyses.[1]

Data Analytic Strategy

Descriptive statistical analyses were conducted using IBM SPSS Statistics 28 to examine normality of variables and identify potential covariates. AUCg and AUCi, as indices of total cortisol output and change in cortisol over the course of the SFP, were used as summary measures to simplify analyses based on the modest subsample with MRI data. However, descriptive data are also provided on cortisol trajectories over the three data collection points in this sample (N = 57) (see Supplemental file). Due to skewness and kurtosis in the AUCg and AUCi variables, scores were log 10 transformed and winsorized to minimize the impact of

[1] Note that left and right amygdala and hippocampal hemispheric analyses yielded similar findings, thus results of total amygdala and hippocampal volumes are presented. See Supplemental Material for hemispheric results.

outliers. Winsorized and transformed scores were used in analyses. For the MRI data, one infant had an outlier [$\geq$ 3 SD from the mean] for total amygdala volume, and the same infant and one additional infant had outliers for total hippocampal volume. These outliers were due to atypical brain anatomy and inhomogeneity resulting in inaccurate segmentations. Following current practice in brain imaging studies (e.g., Chua et al., 2015; Cruz et al., 2020; Rezvan et al., 2015), these outliers were removed and estimated using full information maximum likelihood (FIML) to account for missing data (see Missing Data below).

Potential covariates were assessed by partial correlation and were included in regression and mediation models if they were associated with a relevant dependent or independent variable (AUCg, AUCi, limbic brain volumes, maternal behavior), after controlling for age at assessment. Covariates were also included in regression analyses when prior research/theory suggested their relevance, even if correlations did not reveal significant associations in this sample. Specifically, prior research indicates that saliva collection time can influence cortisol levels (e.g., Hanrahan et al., 2006) and that infant age, sex, and total GMV can impact limbic volumes (e.g., Gilmore et al., 2012). Therefore, regardless of bivariate associations, these variables were included in the regression models.

Linear regression and mediation models were conducted in Mplus Version 8 using maximum likelihood estimation, with bootstrapped standard errors and confidence intervals and FIML to account for missing data, nonnormality, and small sample size (Collins et al., 2001). 95% CIs that do not contain zero are significant at $p < .05$. To test the main study hypotheses, linear regression analyses with relevant covariates were first conducted with level of maternal disrupted interaction as the independent variable and infant AUCg/AUCi as the dependent variables. Follow-up exploratory analyses were then conducted on scores for the five dimensions

of disrupted interaction to further specify components of maternal behavior that might be contributing to the overall finding. Second, linear regression analyses, with relevant covariates, were conducted with infant AUCg/AUCi as the independent variables and infant amygdala or hippocampal volume as the dependent variables. Given rapid changes in infant brain volumes during the first two years (Gilmore et al., 2012), follow-up models were also run to assess whether relations between AUCg/AUCi and limbic volumes were moderated by age at MRI. Finally, mediation models assessed whether maternal disrupted interaction indirectly affected infant amygdala or hippocampal volumes via infant AUCg/AUCi.

Missing Data. There were no missing data for sociodemographic variables. One value (1.8%) was missing for maternal interaction data due to malfunction in video equipment. Missing data for cortisol were as follows: n=4 (7%), n=8 (14%), and n=3 (5.3%) for infant baseline, +20, and +40 min, respectively. Missing cortisol values were due to insufficient saliva collected because of infant fussiness. As noted above, MRI data for two participants had outliers and were removed. Based on Little's MCAR test, these amygdala and hippocampal volumes were missing at random [amygdala: X^2 (12) = 6.49, p = .89; hippocampus: X^2 (12) = 11.30, p = .50]. Thus, amygdala and hippocampal outliers were removed and all missing data were estimated using FIML. FIML estimation utilizes all available data to derive parameter estimates and reduces bias in missing data. Therefore, it is preferred to other methods of handling missing data (e.g., listwise deletion) (Enders & Bandalos, 2001).

Power analyses. Power analyses were computed to assess the effect sizes that could be detected with N = 57 at .80 power. Based on the effect size benchmarks of small $f^2 \geq 0.02$, medium $f^2 \geq 0.15$, and large $f^2 \geq 0.35$ (Selya et al., 2012), regression models with four or five predictors were powered to detect the incremental effect of each variable of interest, significant

at $p < .05$ two-tailed, with effect sizes ranging from $f^2 = 0.1430$ to 0.1431 (Faul et al., 2007). Thus, analyses were powered to detect medium, but not small, effect sizes.

Results

Descriptive Statistics and Assessment of Covariates

Descriptive statistics are shown in Table 1. Preliminary correlation analyses to determine relevant covariates controlled for age at time of MRI (for correlations with limbic volume) or age at time of behavioral assessment (for correlations with infant cortisol or maternal behavior). None of the sociodemographic variables (infant sex, infant gestational age, annual family household income, maternal education, mother living with partner) were associated with limbic volumes, *r*'s = -.24 - .26, *ps* = .10 - .99. GMV was not significantly correlated with amygdala (*r* = .24, *p* = .07) or hippocampal (*r* = .18, *p* = .19) volumes, with age controlled. None of the sociodemographic or time of cortisol collection variables were associated with AUCg/AUCi or maternal disrupted behavior, *r*'s = -.22 - .26, *ps* = .09 - .99. Despite these nonsignificant correlations, based on prior research cited above under Data Analytic Strategy, regression models with limbic volumes as the dependent variables included infant age at MRI, sex, and GMV as covariates and models with total cortisol output as the dependent variable included time of saliva collection as a covariate.

Maternal Disrupted Interaction and Infant Cortisol and Limbic Volumes

Overall maternal disrupted behavior and infant cortisol. Regression analyses were conducted with overall disrupted interaction as the independent variable and infant AUCg/AUCi as dependent variables, controlling for baseline saliva collection time. Maternal overall disrupted interaction was associated with infant total cortisol output (AUCg; β = 0.334, CI = 0.075, 0.580; Table 2) but was not associated with AUCi (Table 2). Figure 1a displays the distribution of

infant AUCg in relation to overall maternal disrupted interaction, with cortisol collection time controlled.

Specific dimensions of disrupted interaction and infant cortisol. Based on the obtained association between overall disrupted maternal interaction and infant cortisol output, follow-up exploratory analyses were conducted to assess whether particular aspects of maternal disrupted interaction were more strongly associated with infant cortisol response than others. Regression analyses controlling for baseline saliva collection time indicated that maternal disorientation was significantly associated with higher infant AUCg ($\beta = 0.435$, CI = 0.162, 0.641; Table 2, Figure 1b), but none of the other dimensions of maternal disrupted interaction approached significance (withdrawal: $\beta = 0.210$, CI = -0.160,0.539; negative-intrusive behavior: $\beta = 0.108$, CI = -0.173,0.396; affective errors: $\beta = 0.059$, CI = -0.243,0.387; role confusion: $\beta = 0.201$, CI = -0.143,0.504). In addition, in a final regression model controlling for maternal disorientation, overall level of maternal disrupted interaction was no longer significantly associated with AUCg ($\beta = 0.101$, CI = -0.273,0.534), indicating that disorientation was driving the overall association and that other aspects of disrupted interaction were not explaining independent variance.

Consistent with the lack of relations between overall level of disruption and AUCi, none of the five sub-dimensions of disrupted interaction were associated with AUCi (disorientation: $\beta = 0.144$, CI = -0.182,0.448; withdrawal: $\beta = -0.263$, CI = -0.579,0.117); negative-intrusive behavior: $\beta = -0.033$, CI = -0.314,0.229; affective errors: $\beta = 0.163$, CI = -0.122, 0.472; role confusion: $\beta = -0.013$, CI = -0.373,0.357).

Overall maternal disrupted behavior and limbic volumes. Regression analyses yielded no significant relation between overall level of maternal disrupted behavior and infant

amygdala or hippocampal volumes, after controlling for infant sex, age at MRI, and GMV (amygdala: β = -0.137, CI = -0.403,0.148; hippocampus: β = -0.067, CI = -0.349,0.197). Given that the subscale for disorientation was also associated with infant AUCg, follow-up regression models were also computed for amygdala and hippocampal volumes, with disorientation as the independent predictor and relevant covariates included. Neither model was significant.

Infant Cortisol Levels and Infant Limbic Volumes

Separate regression analyses were conducted with amygdala and hippocampal volumes as dependent variables and AUCg/AUCi as the independent variables, with infant age, sex, and GMV covaried. Larger AUCg was associated with larger amygdala (β = 0.455, CI = 0.215,0.645) and hippocampal volumes (β = 0.484, CI = 0.206,0.693) (Table 2). Figure 2 displays the distributions of amygdala and hippocampal volumes in relation to infant AUCg, with covariates controlled. AUCi was not associated with limbic volumes (Table 2).

Additional regression models assessed whether age at MRI moderated the effect of cortisol levels on limbic volumes. Age at MRI did not moderate the effect of infant AUCg on either amygdala or hippocampal volume (age by AUCg interaction: amygdala β = 0.400, CI = -4.865, 4.657; hippocampus β = -1.183, CI = -6.301, 4.010) or infant AUCi on amygdala or hippocampal volume (age by AUCi interaction: amygdala β = -0.065, CI = -0.705,0.631; hippocampus β = 0.063, CI = -0.479,0.661). All significant relations described above between infant AUCg and limbic volumes were also significant for left and right hemispheres analyzed separately (see Supplement for hemispheric analyses).

Assessing Infant Cortisol Levels as Mediating Mechanisms

Final analyses examined whether there was an indirect effect of overall maternal disrupted interaction on infant amygdala and hippocampal volumes, mediated through infant

AUCg. These mediation models were examined because there were significant links between maternal disrupted behavior and infant AUCg and between infant AUCg and limbic volumes. Following Preacher and Hayes (2008), significant indirect effects can be present in the absence of significant direct effects. While there was no direct effect of overall maternal disrupted behavior on either amygdala volume or hippocampal volume, as also shown above, there was a marginally significant indirect path from maternal disrupted behavior to infant amygdala volume and a significant indirect path to infant hippocampal volume, through infant AUCg (Figure 3a, b). The indirect effects were positive, indicating that greater maternal disrupted behavior was indirectly associated with larger amygdala volume and larger hippocampal volume through higher infant AUCg.

Given that maternal disorientation accounted for the relation between overall maternal disruption and infant cortisol levels, similar analyses examined whether there was an indirect effect of maternal disorientation on infant amygdala and hippocampal volumes, mediated through infant AUCg. There were also significant indirect paths from maternal disorientation to both infant limbic volumes through infant AUCg (Figure 4 a, b). None of the mediation models with infant AUCi levels were significant (see Supplement for AUCi mediation results).

Discussion

Among rodents, controlled studies have demonstrated that higher early HPA axis activation is related to altered amygdala and hippocampal structure and function (Maras & Baram, 2012; Vyas et al., 2004; Vyas et al., 2006). In addition, low maternal nurturance/maternal unpredictability is a potent stressor associated with altered neurobiological development in rodent pups (Champagne et al., 2008; Drury et al. 2016; Turecki & Meaney 2016). To our knowledge, no work to date has assessed whether similar relations are seen among humans

during the first two years of life when limbic structures are most rapidly developing (Gilmore et al., 2012).

The first finding of the current study was that overall maternal disrupted behavior was related to higher infant cortisol output over the course of a mild stressor, the SFP, at four months. This finding is consistent with previous work linking maternal disrupted interaction or less sensitive interaction with elevated infant cortisol levels over the SFP (Crockett et al., 2013; Erickson et al., 2019; Martinez-Torteya et al., 2014), as well as with a larger literature that consistently finds a relation between more sensitive maternal interaction and more adaptive regulation of infant cortisol over infancy and early childhood (see Atkinson et al., 2016, for review). The specific effect of maternal disoriented interaction, in comparison to other forms of disrupted interaction, is discussed below.

The second major finding was that infant cortisol output was associated with enlarged infant amygdala and hippocampal volumes. These associations were not moderated by age at MRI assessment. Thus, the results portray a general effect of higher cortisol production (but not cortisol reactivity) on larger limbic volumes during the first two years of life. This link among human infants is novel but is consistent with animal studies of the amygdala, which show increases in amygdala volume in relation to early stress or corticosterone administration (Vyas et al., 2004; Vyas et al., 2006).

Our finding that cortisol output was associated with increased hippocampal volume differs from some findings in both animal and human literatures. In animal studies, HPA activity (including CRH, corticosterone elevations) has more often been related to *reduced* hippocampal volume (e.g., Chen & Baram 2016; Lee et al., 2009). However, some animal research has failed to find a negative association between HPA activity and hippocampal volume (Leverenz et al.,

1999; Tuvnes et al., 2003). In addition, a meta-analysis of research on humans who experienced childhood abuse revealed mixed findings, with reduced hippocampal volume seen among adults exposed to childhood abuse, but no differences in hippocampal volume seen among children exposed to abuse (Reim et al., 2015). Notably, however, our findings are consistent with human studies of younger children, which have shown associations between higher cortisol levels and increased hippocampal volumes (Wiedenmayer et al., 2006) and between exposure to insensitive parenting in infancy and increased hippocampal volumes across ages (Bernier et al., 2019; Lee et al., 2019; Khoury et al., 2019; Rao et al., 2010; Rifkin-Graboi et al., 2015; but see Kok et al., 2015; Luby et al. 2012). In addition, greater maternal postnatal anxiety (known to be correlated with elevated maternal and infant cortisol) is associated with greater infant right hemisphere hippocampal growth (Qiu et al., 2013).

One possible mechanism that may be involved in linking insensitive maternal behavior to enlarged infant hippocampal volumes among human infants is heightened infant attention to predicting maternal behavior. A large body of attachment research (Ainsworth et al., 1978; Cassidy & Shaver, 2016) has documented that infants adapt their attachment behavior to a range of caregiving contingencies by the end of the first year of life, based on their developed expectancies regarding caregivers' likely responses to bids for proximity, contact, and comfort. Such expectations, termed 'working models of attachment', are likely to require involvement of the hippocampus in retaining and retrieving expectations regarding caregiver behavior. Caregivers who are less consistently responsive tend to have infants who increase (hyperactivate) their distress signaling and contact-seeking (e.g., Ainsworth et al., 1978). We have previously suggested that both the amygdala and the hippocampus may become enlarged in the context of the sustained hypervigilance to caregiver availability elicited by more pronounced disrupted

caregiver behavior (Khoury et al., 2019; Lyons-Ruth et al., 2016). Supporting this possibility, prior research in preschoolers has shown that lower maternal sensitivity was associated with memory bias towards negative stimuli, and that such memory bias was linked to larger hippocampal volume (Rifkin-Graboi et al., 2023). More research on aspects of caregiving and limbic volumes will be needed to evaluate this possibility. Below we further discuss possible models for integrating animal and human work on early stress responding.

Because overall cortisol output (AUCg) can be associated with a variety of cortisol trajectories, we descriptively examined cortisol levels over time (Supplementary Figure 1) to show that larger limbic volumes were associated with higher cortisol levels 20-minutes and 40-minutes post-stressor, whereas lower limbic volumes were associated with decreased output post-stressor. Similarly, higher levels of disoriented maternal behavior were associated with higher cortisol values post-stressor (Supplementary Figure 2). This pattern of continued cortisol secretion over the SFP, in relation to both maternal disoriented interaction and enlarged infant limbic volumes, is consistent with the pattern of continued cortisol elevation shown in relation to maternal risk factors in other studies, including maternal childhood maltreatment (Khoury et al., 2021) and less sensitive maternal care (Crockett et al., 2013; Erickson et al., 2019; Martinez-Torteya et al., 2014). These studies provide converging evidence that the higher infant AUCg values observed here represent a risk-related stress response pattern to the SFP.

Finally, the results of mediation models suggest that a) disoriented maternal interaction was related to infant stress hormone release which, b) in turn, was associated with larger infant limbic volumes, and c) disoriented maternal interaction was indirectly associated with larger infant limbic volumes through elevated infant cortisol levels. This pattern of findings provides evidence for one potential biological mechanism linking maternal behavior to alterations in

infant neurodevelopment. In the current study, both maternal interaction and infant cortisol levels were assessed prior to the assessment of infant limbic volumes. Therefore, mediation was assessed with limbic volumes as the outcomes. However, given the correlational design and the assessment of each variable at only one point in time, other models of mediation remain plausible, including effects of limbic volumes on cortisol levels and effects of infant stress levels on maternal behavior. In addition, limbic volumes could moderate the impact of maternal behavior on infant cortisol responses, as has been shown in relation to other child outcomes (e.g., Nolvi et al., 2020a, 2020b; Rifkin-Graboi et al., 2019). These potential alternative models should be explored in future research. Most importantly, the pattern of effects found here calls for future randomized intervention studies to assess potential causal pathways linking disrupted maternal behavior to infant limbic volumes through cortisol output.

It is important to consider how the current results in human infancy might be integrated both with animal research showing *increased* limbic volumes in response to stress and with work on adults showing *decreased* limbic volumes associated with childhood abuse (Mclaughlin et al., 2019; Teicher et al., 2016). There are at least two potential models that might integrate these diverging findings. First, consistent with the allostatic load model, the effect of HPA activation on limbic volumes may change over time, with early HPA activation in response to maternal disrupted caregiving resulting in larger limbic volumes, but chronic disrupted caregiver behavior leading to blunted HPA responding and subsequent limbic atrophy. In this model of allostatic load, prolonged exposure to threat and concomitant continued cortisol release place allostatic load on the body that can lead to downregulation (blunting) of the HPA-axis (McEwen, 1998). Over time, this downregulation is thought to lead to reductions in limbic volumes.

However, it is also important to consider the possibility that there may be developmental changes in the types of stressors that are most salient to HPA activity at different points in development. Sullivan and colleagues (e.g., Moriceau & Sullivan, 2006; Opendak & Sullivan, 2016) have shown that there is a hyporesponsive period to aversive (pain-related) maternal stimuli among rodent pups. Using an abusive mother paradigm (pairing maternal odor with electric shock), Opendak and Sullivan (2016) found that shock-associated maternal odor does not activate the HPA axis prior to weaning. This hyporesponsive period is thought to protect pups' early bonding to the mother, regardless of abusive behavior, and begins to diminish around the time of weaning. However, rodent studies have also demonstrated that, prior to weaning, low maternal nurturance is a reliable stressor for the rodent pup. Thus, maternal unresponsiveness may be more developmentally salient very early in life, while aversive maternal behavior may become more salient after the offspring is less dependent on maternal care.

If cues signaling lack of care are privileged by the threat detection system in early infancy, this would call for an expanded 'developmental salience' model of threat, in which both threat of lack of care and threat of attack are separately modeled and thought to be developmentally sequenced. One strength of this model is that these two forms of threat require different defensive/adaptive responses (Lyons-Ruth et al., 2016). The often-cited fight, flight, or freeze behaviors that are adaptive responses to threat of attack would be highly maladaptive as responses to threat of lack of care (abandonment) by the caregiver. Across species, infants respond to lack of care by calling and contact-seeking to restore proximity to the caregiver (e.g., Ainsworth et al., 1978; Sanchez et al., 2015; Turecki & Meaney, 2016). Thus, across species, infants mount a stress response to low maternal care but respond to that threat with different adaptive behaviors than those elicited by threat of attack.

Notably, the specific association of infant cortisol levels with disorientation, but not other components of disrupted maternal behavior, may also be consistent with such a framework. Maternal negative, hostile, or intrusive behavior has been the most widely studied aspect of problematic parenting, with negative effects spanning across development (e.g., Lyons-Ruth et al., 1999; Pinquart et al., 2017; Smith et al., 2004). In the larger MIND sample, negative-intrusive maternal behavior was specifically associated with infant negative affect at four months of age (Khoury et al., 2022). Thus, we might expect that maternal negative-intrusive behavior would be similarly associated with infant cortisol levels, but that was not the case. Notably, maternal role-confused behaviors and affective communication errors also index more actively aversive behaviors, and those were also unrelated to infant cortisol levels.

In contrast, maternal disorientation is defined by indices of emotional disconnection/affective distortion (odd affect, false affect, frenetic interaction) (Lyons-Ruth et al., 1999), and maternal withdrawal is defined by indices of physical/behavioral disengagement from the infant (leaning away; interacting silently). In the early months of life, the exchange of mutually contingent affective signals is essential to caregiver-infant face-to-face communication (e.g., Jaffee et al., 2001). Thus, it is notable that the dimension of maternal behavior most strongly associated with infant cortisol output was the dimension indexing disconnected (odd or false) affect/behavior that blocks effective affective communication between mother and infant.

Disoriented caregivers appear uncomfortable, awkward, and quick to disengage in their attempts to relate to their infants (Lyons-Ruth et al., 2009). Although the substantial differences between human and rodent caregiving make comparisons difficult, disoriented maternal behavior may be consistent with rodent models of low maternal nurturance/maternal unpredictability, where the rodent mother is either disinclined or anxious and distracted from nurturing her infant

pups (Drury et al., 2016; Turecki & Meaney, 2016). Maternal disorientation is associated with maternal psychopathology, maternal history of childhood maltreatment, and aberrant infant attachment behavior (Khoury et al., 2022; Lyons-Ruth et al., 1999; Lyons-Ruth et al., 2009; Lyons-Ruth et al., 2019). The current results extend these findings to suggest that increased infant cortisol levels and enlarged limbic volumes may also be correlates of early disoriented maternal care.

Strengths and limitations

This study has several strengths, including assessments of infant cortisol responses to a relational stress paradigm, observational coding of maternal behavior, and brain imaging during the first two years of life. However, findings should be interpreted in the context of study limitations. This study included a relatively small sample of infants assessed between 4 and 25 months of age. Concerns are justified regarding the replicability of small sample results in imaging studies. Given that infants are difficult to image without sedation beyond the neonatal period, meta-analyses of a series of smaller studies may be needed to arrive at reliable effect sizes in infancy. Larger studies often require methodological trade-offs, in particular the use of parental self-reports or simplified assessments of caregiving. Intensive multimethod studies of smaller samples can provide valuable 'proof of concept' studies to identify critical variables for inclusion in more costly large-scale studies. NEED highlight for Reviewer 3 comment 3?

In addition, because all data were correlational, direction of causality cannot be inferred. Mediation models, in particular, need replication to be confident of their replicability. Also importantly, MRI data collection was cross-sectional, so no conclusions can be drawn regarding changes in brain volumes over time. Finally, prenatal and gestational influences on maternal behavior and infant neurobiology were not assessed here, although they likely contribute to these

early developmental pathways (Moog et al., 2022). Understanding how the postnatal pathways identified here relate to prenatal risk factors will be an important priority for future work.

Conclusion

This research addresses a critical gap in our knowledge of neurobiological development in infancy by demonstrating relations between maternal disrupted behavior, infant cortisol release, and infant limbic volumes. Our results largely confirm the relevance of the large body of animal studies to early human development, with disrupted caregiving associated with elevated infant cortisol output in response to stress, and elevated infant cortisol output associated with increased limbic volumes during the first two years of life. Mediation models further suggest that differences in maternal behavior may indirectly affect infant limbic volumes through infant cortisol levels. While needing replication, these results also offer the possibility that early evidence-based interventions shown to reduce disrupted caregiving (e.g., Yarger et al., 2020) may have the potential to alter these worrisome neurobiological outcomes in infancy.

Declaration of Competing interests: The authors declare that they have no competing financial or other interests that could appear to influence the current work. **Data Availability:** The data that support the findings of this study are available from the corresponding author upon reasonable request. **Ethical Approval Statement**: This study was approved by the Partners Healthcare Institutional Review Board [IRB Protocol #: 2014P002522] and parents provided written informed consent. **Funding Source and Acknowledgments:** This work was supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development Grant # R01HD079484 to K. Lyons-Ruth, M. H. Teicher, P.E. Grant, M. Bosquet Enlow. We would like to thank the families whose interest and participation made this work possible. We would also like to express our appreciation for the hard work and dedication of the study staff who were responsible for subject recruitment and behavioral data acquisition, including Carrie Heldstedt, Rachael Phillips, Molly Rothenberg, Ilana Shiff, Mariya Patwa, Molly Cunningham, Lina Dimitrov, Mallika Rajamani, Danielle Farrell, Sarah Immelt, and Sommer Jaber.

Table 1

Sociodemographic characteristics and descriptive statistics

	M (SD) or % (N)	Range
Gestational age (weeks)	M = 39.48 (SD = 1.60)	36 - 42
Infant age (months) at SFP	M = 4.54 (SD = 0.85)	3.16 - 7.36
Infant age (months) at MRI	M = 11.74 (SD = 6.12)	4.00 - 25.00
Infant sex		
Female	51% (n = 29)	
Male	49% (n = 28)	
Infant race/ethnicity		
White/Non-Hispanic	57.9% (n=33)	
Black	8.8% (n=5)	
Asian	1.8% (n=1)	
Hispanic	5.3% (n=3)	
Multi-racial	26.3% (n=15)	
Infant minority race/ethnicity		
Minority race/ethnicity	42.1% (24)	
White/non-Hispanic	57.9% (33)	
Maternal relationship status		
Living with partner	49 (86%)	
Not living with partner	8 (14%)	
Maternal education		
High school	14.0% (n=8)	
Associate degree	8.8% (n=5)	
Bachelor's degree	24.6% (n=14)	
Master's degree	35.1% (n=20)	
Doctoral degree	17.5% (n=10)	
Annual family income		
$0 to $15,000	7.0% (n=4)	
$16,000 to $25,000	3.5% (n=2)	
$26,000 to $50,000	10.5% (n=6)	
$51,000 to $75,000	26.3% (n=15)	
$76,000 to $100,000	17.5% (n=10)	
$101,000 to $150,000	17.5% (n=10)	
$151,000 +	17.6% (n=10)	
Maternal disrupted interaction		
Overall level of disruption	M= 3.05 (SD= 1.70)	1 - 7
Affective communication errors	M= 2.77 (SD= 1.54)	1 - 6
Role confusion	M= 2.73 (SD= 1.67)	1 - 6
Disorientation	M= 3.70 (SD= 1.51)	1 - 6

Negative/ intrusive behavior	M= 1.82 (SD= 1.19)	1 - 6
Withdrawal	M= 3.70 (SD= 1.67)	1 - 7
Infant AUCg (nmol/L)	M= 45.42 (SD= 3.61)	41.69 - 58.82
Infant AUCi (nmol/L)	M= -0.68 (SD= 3.28)	-8.17 – 6.10
Amygdala volume (mm3)	M = 2387.37 (SD = 748.41)	1256 - 4795
Hippocampal volume (mm3)	M = 6099.55 (SD = 1221.24)	3829 - 9976

Note: Outliers removed from amygdala and hippocampal volumes. Infant AUCg/AUCi cortisol is log-transformed and winsorized. N = 47 for AUCg/AUCi, N = 56 for amygdala volume, N=55 for hippocampal volume, N= 56 for maternal disrupted interaction. All missing data estimated in final models.

Table 2

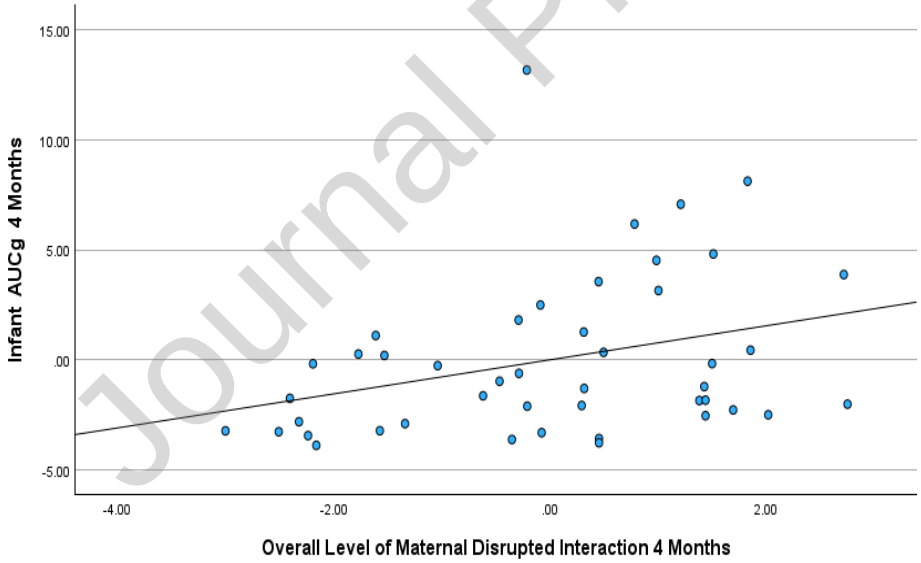
Regression coefficients from separate regression models examining associations between maternal overall disrupted behavior/ maternal disorientation and infant total cortisol output (AUCg)/change in cortisol (AUCi) and between infant cortisol levels and infant amygdala volume and infant hippocampal volume.

	AUCg				AUCi			
	B	**SE**	**β**	**95% CI**	**B**	**SE**	**β**	**95% CI**
Maternal overall disrupted behavior → Infant cortisol								
Saliva collection time	-0.288	0.305	-0.147	-0.408, 0.182	-0.053	0.356	-0.030	-0.418, 0.341
Maternal disrupted behavior	0.721	0.275	0.334*	0.075, 0.580	-0.123	0.281	-0.063	-0.328, 0.232
Maternal disorientation → Infant cortisol								
Saliva collection time	-0.302	0.314	-0.154	-0.439, 0.131	-0.136	0.351	-0.076	-0.451, 0.291
Maternal disorientation	0.943	0.335	0.435*	0.162, 0.641	0.284	0.320	0.144	-0.182, 0.448
Infant cortisol → Infant amygdala volume								
GMV	0.002	0.001	0.320	-0.041, 0.774	0.023	0.014	0.361	-0.035, 0.795
Age at MRI	0.000	0.001	0.054	-0.457, 0.467	-0.001	0.001	-0.141	-0.646, 0.345
Sex	0.222	0.194	0.150	-0.120, 0.398	0.185	0.226	0.125	-0.198, 0.420

Cortisol	0.094	0.032	0.455**	0.215, 0.645	0.036	0.038	0.156	-0.182, 0.456
Infant cortisol → Infant hippocampal volume								
GMV	0.001	0.002	0.113	-0.262, 0.651	0.001	0.002	0.112	-0.273, 0.605
Age at MRI	0.002	0.001	0.244	-0.251, 0.646	0.001	0.002	0.083	-0.403, 0.529
Sex	0.472	0.308	0.195	-0.072, 0.442	0.416	0.365	0.172	-0.137, 0.466
Cortisol	0.166	0.061	0.484**	0.206, 0.693	0.029	0.062	0.079	-0.234, 0.374

Note. N = 57; AUCg = Area under the curve with respect to ground, or total cortisol output; AUCi = Area under the curve with respect to increase, or change in cortisol; GMV= Grey matter volume CI's that do not contain zero are significant at $p < .05$.

1a.



1b.

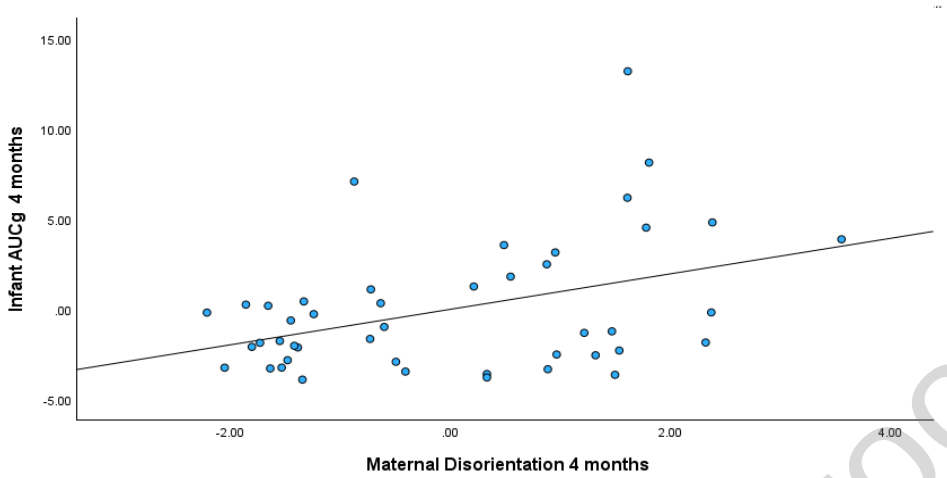
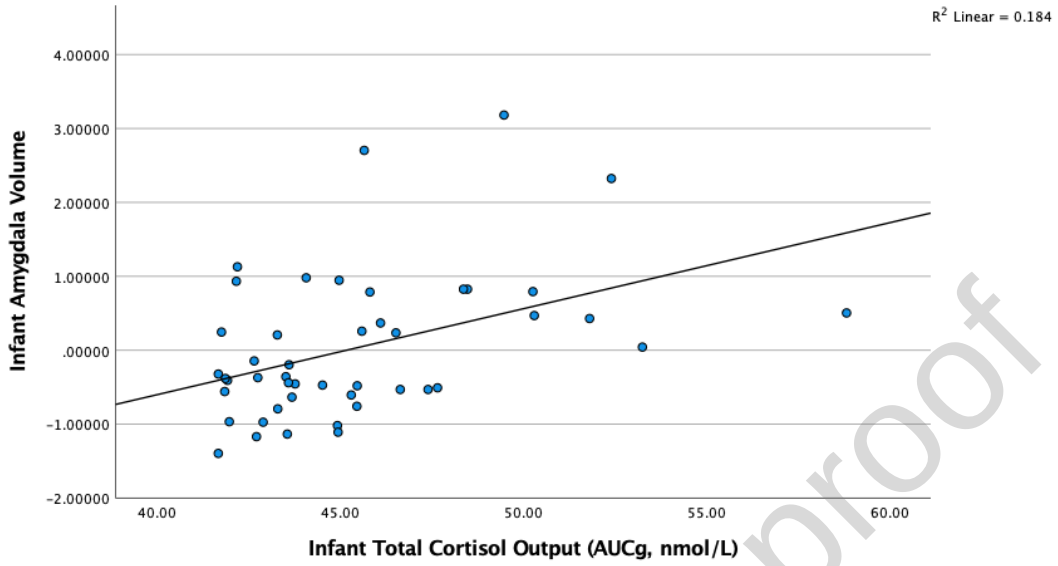


Figure 1a, b.

Distribution of infant cortisol levels (AUCg) at four months as a function of overall level of maternal disrupted interaction and the subscale for maternal disorientation, adjusted for time of cortisol collection.

Note. Plots show standardized residuals. AUCg was log-transformed and winsorized. AUCg metric is nmol/L. Regression data presented without estimation of missing data, with cortisol collection time controlled; Plotted data reflect N = 45, with missing data on maternal interaction for one mother and missing data on one or more of the three time points needed for computing AUCg for 10 infants. Final regression analyses presented in text were conducted using FIML (N=57).

2a.



2b.

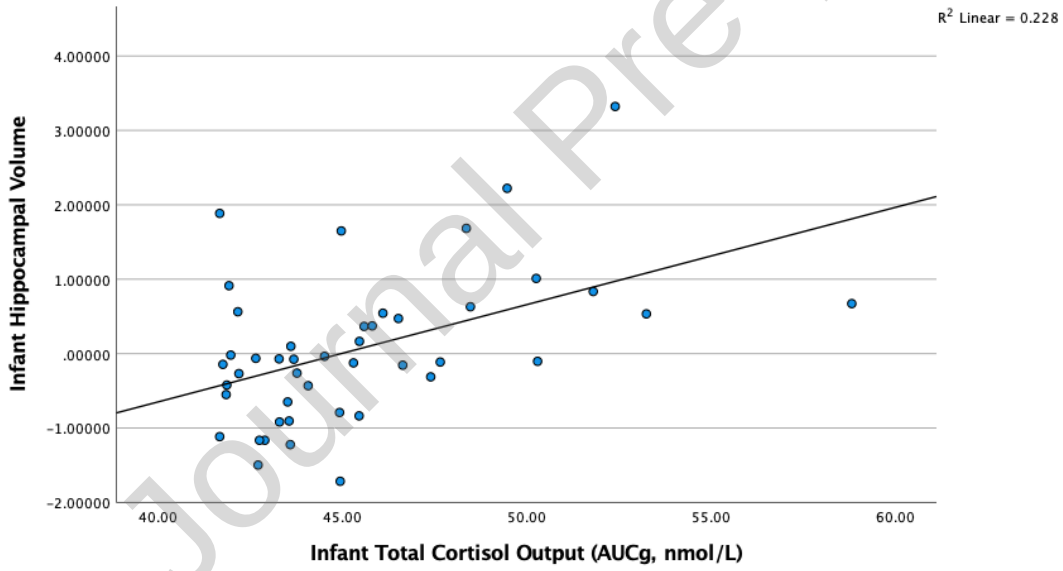


Figure 2a,b.

Distribution of infant amygdala and hippocampal volumes as a function of infant cortisol output (AUCg), adjusted for effects of age at MRI, sex, and GMV.

Note. Plots show standardized residuals. Limbic volume metric is $mm^{3.}$ Regression data presented without estimation of missing data, with age, sex, and GMV controlled; Plotted data reflect missing data on infant cortisol at one or more of the three time points contributing to AUCg for 10 infants and removal of one outlier for amygdala volume and two outliers for hippocampal volume, amygdala N = 46, hippocampus N = 45. AUCg was log-transformed and winsorized. Final regression analyses were conducted using FIML (N=57).

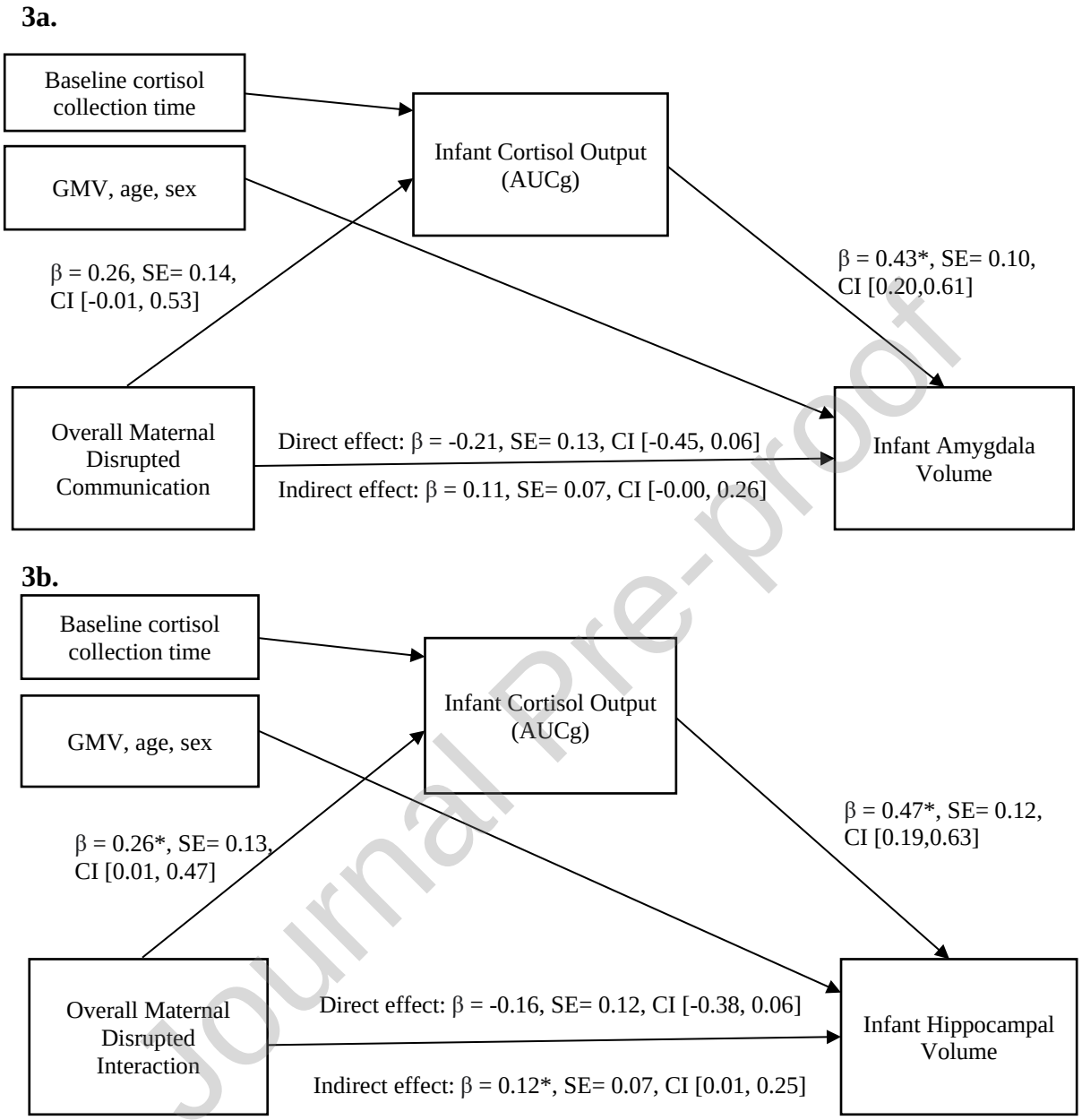
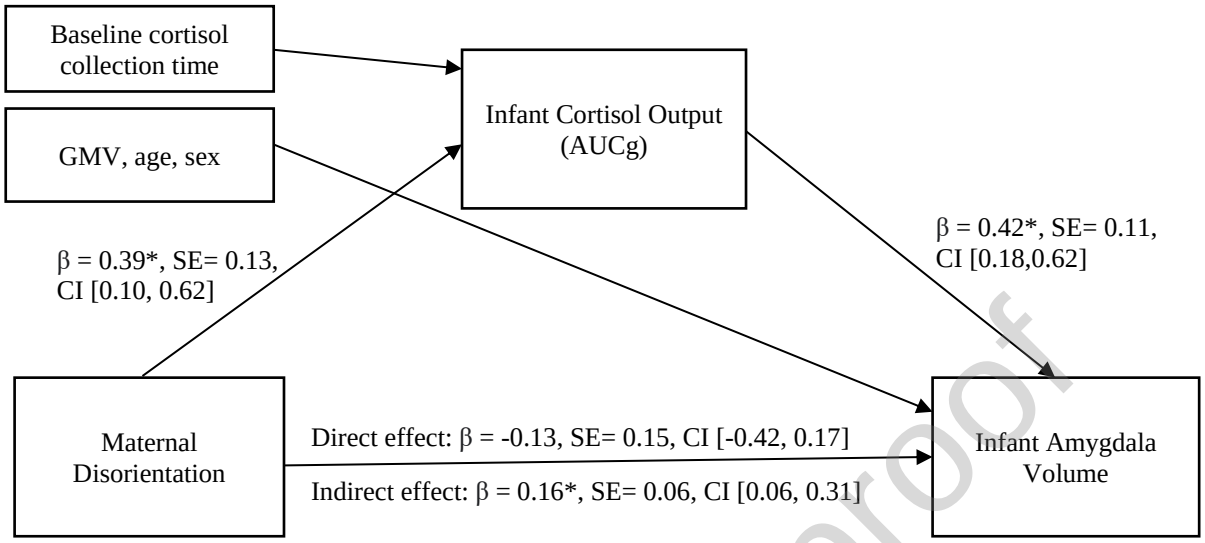


Figure 3a,b.

Standardized coefficients and confidence intervals from mediation models with overall maternal disrupted interaction predicting infant amygdala volume (Figure 2a) and infant hippocampal volume (Figure 2b) through infant cortisol output.

Note: CI's that do not contain zero are significant at $p < .05$. N = 57.

4a.



4b

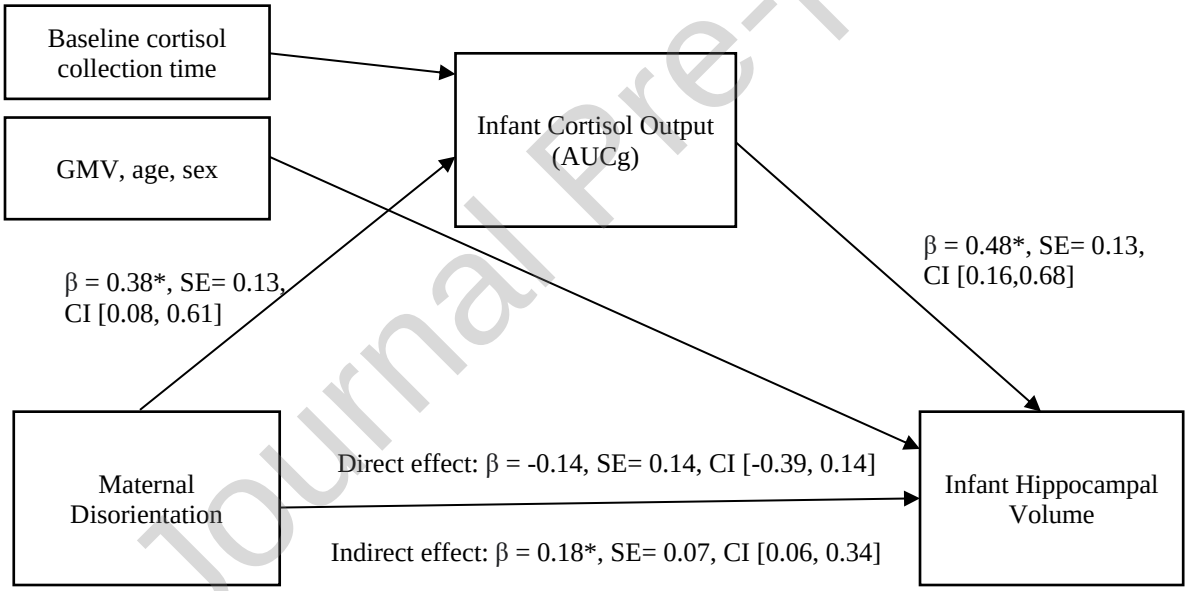


Figure 4a,b.

Standardized coefficients and confidence intervals from mediation models with maternal disorientation predicting infant amygdala volume (Figure 3a) and infant hippocampal volume (Figure 3b) through infant cortisol output.

Note: CI's that do not contain zero are significant at $p < .05$. N = 57.

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Conflict of interest:
The authors do not have any conflicts of interest to disclose.

Highlights

- Higher cortisol associated with enlarged amygdala and hippocampal volumes in infancy
- Maternal behavior indirectly linked to limbic volumes through elevated infant cortisol
- Results in human infants are consistent with animal research