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Developmental pathways to amygdala-prefrontal function and internalizing symptoms in adolescence

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Early life stress (ELS) and function of the hypothalamic-pituitary-adrenal axis predict later psychopathology. Animal studies and cross-sectional human studies suggest that this process might operate through amygdala–ventromedial prefrontal cortex (vmPFC) circuitry implicated in the regulation of emotion. Here we prospectively investigated the roles of ELS and childhood basal cortisol amounts in the development of adolescent resting-state functional connectivity (rs-FC), assessed by functional connectivity magnetic resonance imaging (fcMRI), in the amygdala-PFC circuit. In females only, greater ELS predicted increased childhood cortisol levels, which predicted decreased amygdala-vmPFC rs-FC 14 years later. For females, adolescent amygdala-vmPFC functional connectivity was inversely correlated with concurrent anxiety symptoms but positively associated with depressive symptoms, suggesting differing pathways from childhood cortisol levels function through adolescent amygdala-vmPFC functional connectivity to anxiety and depression. These data highlight that, for females, the effects of ELS and early HPA-axis function may be detected much later in the intrinsic processing of emotion-related brain circuits.

Dysregulation of the HPA axis is related to many adverse physiological and psychological outcomes[1]. Cortisol, a principal hormone end product of HPA-axis activity, has a direct influence on brain circuits implicated in the regulation of emotion, and these circuits in turn regulate HPA-axis activity. Increased cortisol levels early in life are associated with poor emotion regulation, less social competence, and later symptoms of anxiety and depression[2,3]. These individual differences may be primed by exposure to ELS, and the timing and magnitude of these effects can vary by gender[4].

There are multiple distinct lines of research that investigate questions related to how ELS and the HPA-axis work together to influence emotion-related brain circuits and the development of psychopathology. For example, our group finds that children exposed to elevated levels of maternal stress in infancy are more likely to display higher afternoon basal cortisol levels later in childhood, which, in turn, is associated with increased mental health symptoms two years later[4]. Other work suggests that aspects of ELS may sensitize the neural circuitry that regulates HPA-axis feedback resulting in atypical basal cortisol levels (for example, heightened or blunted cortisol function[2,3]) as well as structural and functional changes in brain regions involved in affective reactivity and regulation, such as the amygdala[5] and vmPFC[6,7]. The amygdala-vmPFC pathway may be of particular importance in these associations given nonhuman primate work that demonstrates that the prefrontal cortex has a dense collection of glucocorticoid receptors[8]. Elevated expression and concentrations of corticosteroid releasing hormone receptors in the amygdala and other limbic regions are also linked to both ELS and anxiety-like behaviors in nonhuman primate and other animal studies, particularly in females[9–12]. In fact, task-based fMRI studies of emotion regulation suggest that the vmPFC is involved in top-down regulation of the amygdala, and functional coupling of the amygdala and vmPFC is related to trait anxiety in both task-related and resting-state studies[13–15].

Recent work implicates ELS in morphometric differences in the amygdala and the PFC[5,16]. Strength of white matter tracts connecting these regions may also be inversely associated with anxiety symptoms in adults[17], suggesting evidence of disruptions in structural as well as functional connectivity. Dysregulated diurnal cortisol patterns are associated with the functional interplay among these brain regions[18]. Thus, although interactions between ELS and HPA-axis function are known to affect brain structure and behavior, the roles of ELS and neuroendocrine processes in the development of the functional interplay, or connectivity, of the amygdala-vmPFC circuit and its associations with internalizing problems later in life are not yet clear[19].

Resting-state fcMRI is an excellent metric for probing the functional integrity of the amygdala-vmPFC circuit[20]. This metric is postulated to reflect critical individual differences in spontaneous brain activity that can be captured independent of task demands and has evidenced patterns of functional connectivity consistent with the white matter structure of the brain[21,22]. Highly anxious individuals exhibit reduced amygdala-vmPFC rs-FC[14], whereas higher levels of current basal cortisol are associated with less amygdala-vmPFC connectivity in adults[15]. These differences in the amygdala-vmPFC circuit may be particularly relevant to anxiety given the role this pathway is postulated to have on the down-regulation of threat- or fear-related activity in the amygdala[6,13,18].

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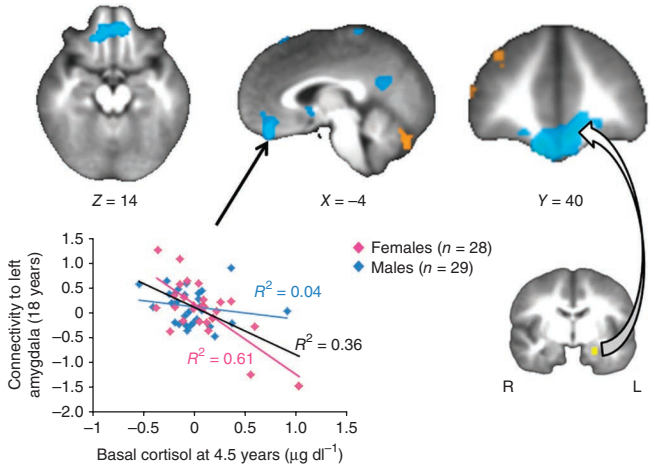

Figure 1 Correlation between late-afternoon cortisol at age 4.5 years and rs-FC to the left amygdala at 18 years. Connectivity between the left amygdala and vmPFC is significantly negatively associated with childhood cortisol ($R^2 = 0.36$, FDR-corrected $P = 0.01$). This effect is driven entirely by data for females ($R^2 = 0.61$, FDR-corrected $P = 0.01$).

In addition, the vmPFC may also mediate perceptions of stress-controllability in behavioral inhibition, particularly in females[23,24].

Resting-state data from samples of individuals with major depressive disorder reveal hyperactivity in the vmPFC and limbic structures (for example, amygdala and bed nucleus of the stria terminalis)[25] as well as increased connectivity between medial prefrontal regions (particularly the vmPFC) and other networks, including an affective network to which the amygdala is interconnected[26]. Such data are consistent with prolonged and persistent experience of negative emotion and affect dysregulation, which may be associated with treatment-resistant symptomatology and disruptions in the stress-response circuitry of the brain[27]. However, given the high comorbidity of anxiety and depression, as well as the consistent involvement of amygdala-vmPFC circuitry in studies of anxiety and depression[28], systematic disentangling of the role of vmPFC-amygdala circuitry in anxious and depressive symptoms is required. There is very limited evidence to address this issue, and in a rare example of rigorous parsing of anxious and depressive symptoms in adolescence, researchers observe reliable differences in amygdala activation as a function of specific task requirements[29]. However, to the best of our knowledge, no research to date has examined the differential relations between amygdala-vmPFC resting-state connectivity and anxious and depressive symptoms or has explored how these associations are impacted by ELS and childhood HPA-axis function.

RESULTS

The present study

In this study we investigated (i) the associations between ELS, early neuroendocrine function and adolescent rs-FC, and (ii) the differential association of adolescent rs-FC as well as ELS and early neuroendocrine function, with concurrent symptoms of anxiety and depression. To affirm that any associations observed were due to early experience, we also examined adolescent life stress and afternoon basal cortisol from this period. Our central prediction was that ELS would be associated with the development of heightened late-afternoon childhood basal cortisol levels, and that both ELS and childhood cortisol would predict variations in adolescent rs-FC. Specifically, we predicted that ELS and cortisol would be positively correlated, and that both would be inversely related to connectivity estimates between the amygdala and vmPFC, which would reflect basal abnormalities in this key regulatory circuit. Following from this, we also predicted that resting-state amygdala-vmPFC rs-FC would be associated with concurrent adolescent internalizing symptoms, especially anxiety. Additional analyses explored whether amygdala-vmPFC rs-FC mediated the association of childhood cortisol and internalizing symptoms. Finally, based on gender differences found in studies summarized above, we investigated gender differences in all analyses.

In the present study, we acquired structural MRI and resting-state fMRI data, along with self-reported psychiatric symptoms, from 57 (28 female, mean age = 18.44, s.d. = 0.19 years) participants selected from the Wisconsin Study of Families and Work (WSFW)[4], a prospective longitudinal study of a community sample beginning during the prenatal period. We computed rs-FC of the amygdalae at age 18 years by first defining masks of the left and right amygdala using previously developed[30] probabilistic maps in Talairach space[31]. We extracted the averaged preprocessed signal time courses from each amygdala and regressed them with resting-state time courses from each voxel in the rest of the brain. Then we regressed connectivity values across participants against measures of mean salivary basal cortisol obtained in childhood at age 4.5 years, which had been collected on three consecutive days at a preselected target time between 3 p.m. and 7 p.m. (mean collection time = 5:56 p.m., s.d. = 88 min). We chose this time as most likely to reflect childrens' experiences of stress during the day[32]. Cortisol was then assayed using the Pantex Radioimmunoassay kit, modified for salivary data. Mean late afternoon basal cortisol values were $\log_{10}$ transformed and residualized for time of collection and concomitant use of over-the-counter medication[4]. We measured ELS exposure using a composite measure of early maternal stress that we computed using maternal reports of depressive symptoms, parenting stress, marital conflict, role overload and financial stress, averaged over assessments conducted when children were 1 month, 4 months and 12 months old[4]. Current adolescent psychiatric symptoms were indexed via self-report on the MacArthur health and behavior questionnaire[33]. We computed the general anxiety and depressive subscales, and used the externalizing subscale as a control variable in all analyses of psychiatric symptoms. Adolescent recent life stress was measured with a self-report measure derived from the Adolescent Perceived Events Scale[34] and the Life Events Survey[35]. Finally, all data were winsorized to within 3 s.d. of the mean to normalize the distribution in the subsample.

Initial analyses examined voxel-wise functional connectivity estimates using the left amygdala seed (associations using the right amygdala were similar). Results indicated significant ($P < 0.05$) positive correlations between left amygdala rs-FC and the right

Table 1 Resting-state functional connectivity estimates

Identified clusters	Talairach coordinates of peak voxel			Peak *t*-statistic	Volume (mm^3)
	x	*y*	*z*		
vmPFC	10	38	−24	−5.97	978
Posterior cingulate	−2	−54	22	−4.74	321
Cerebellum					
L posterior	−16	−78	−42	4.74	357
R posterior	16	−76	−42	5.06	318
R anterior	36	−44	−54	−5.95	266
L anterior	−34	−56	−56	−5.37	210

Estimates are with left amygdala region of interest as predicted by childhood late afternoon basal cortisol levels ($n = 57$). FDR-corrected with $q < 0.05$ ($P < 7 \times 10^{-4}$).

Table 2 Pearson-*r* bivariate correlation statistics

Average values of predictors	Childhood cortisol			Adolescent amygdala-vmPFC rs-FC		
	Females *r* value	Males *r* value	Total *r* value	Females *r* value	Males *r* value	Total *r* value
ELS	0.45*	0.08	0.32*	–0.36#	0.07	–0.22
Childhood cortisol level				0.78**	0.19	0.60**
Adolescent life stress	–0.13	–0.19	–0.13	0.16	–0.10	0.05
Adolescent cortisol level	0.31	–0.02	0.22	–0.21	–0.13	–0.21

$n = 57$ (28 female). #$P < 0.10$, *$P < 0.05$ and **$P < 0.001$. There were no significant zero-order two-tailed correlations between ELS and adolescent life stress (females: $r = –0.13$; males: $r = –0.06$; total: $r = –0.08$).

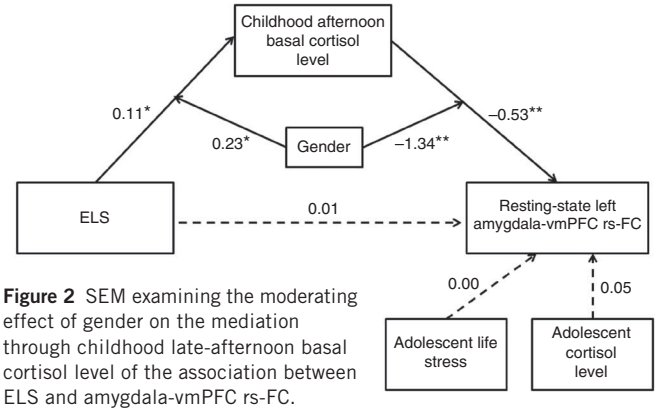

Figure 2 SEM examining the moderating effect of gender on the mediation through childhood late-afternoon basal cortisol level of the association between ELS and amygdala-vmPFC rs-FC. The SEM demonstrated good fit: $\chi^2 = 1.89$, $P > 0.05$; root mean square error of approximation (RMSEA) = 0.00; standardized root mean square residual (SRMR) = 0.03; comparative fit index = 1.00. Paths are marked with unstandardized coefficients. *$P < 0.05$, **$P < 0.01$.

amygdala, dorsomedial and ventrolateral PFC, ventral striatum, superior temporal gyrus and vmPFC, consistent with earlier studies[10].

ELS, childhood cortisol amounts and adolescent rs-FC

Voxel-wise analyses with the left amygdala seed revealed no significant associations between ELS and rs-FC between the amygdala and any other brain regions ($P > 0.05$). However, as predicted, childhood cortisol was negatively correlated with rs-FC estimates between the left amygdala and a voxel cluster in the vmPFC (peak *t*-statistic: $t(56) = –5.97$, $R^2 = 0.36$, false detection rate (FDR)–corrected $P < 0.01$). Higher late-afternoon cortisol levels during childhood were associated with decreased amygdala-vmPFC rs-FC 14 years later (**Fig. 1** and **Table 1**). We detected no voxel-wise relations between amygdala-vmPFC rs-FC and either adolescent basal cortisol amount or current life stress, suggesting that the observed effects were specific to childhood late-afternoon basal cortisol function, not current stress or cortisol amounts.

Bivariate Pearson-*r* (two-tailed) correlations for the total sample revealed a significant positive association between ELS and childhood late-afternoon cortisol amount, and a significant inverse association between childhood cortisol amount and adolescent amygdala-vmPFC rs-FC ($P < 0.05$; **Table 2**). When we considered the results separately for males and females, these effects were largely specific to females. Moreover, only females demonstrated a detectable, but marginally significant ($P < 0.10$), inverse relationship between ELS and amygdala-vmPFC rs-FC, with higher ELS exposure linked to lower functional connectivity.

These results, and the temporal ordering of the variables, suggested that the associations of ELS and childhood cortisol with adolescent amygdala-vmPFC rs-FC might reflect a moderated mediational process. Thus, we constructed a structural equation model (SEM) using Mplus[36] (version 5.2) to determine whether childhood cortisol mediated the association between ELS and amygdala-vmPFC rs-FC and whether gender moderated this association. In addition, we included adolescent recent life stress and current afternoon basal cortisol as controls to confirm that the observed effects were indeed specific to early stress and childhood basal cortisol function. This model (**Fig. 2**) showed excellent fit and accounted for 39.1% of the variance in rs-FC. There was no direct effect of ELS on adolescent amygdala-vmPFC rs-FC, and the mediating effect of childhood cortisol was marginally significant (indirect path estimate = –0.06, $P < 0.10$). Gender significantly moderated both pathways ($P < 0.05$). For females only, higher levels of ELS predicted increased levels of childhood afternoon basal cortisol, which, in turn, predicted decreased amygdala-vmPFC functional connectivity.

Adolescent rs-FC and internalizing symptoms

Bivariate Pearson-*r* (two-tailed) correlations revealed no significant associations between adolescent amygdala-vmPFC rs-FC and concurrent anxiety or depressive symptoms before controlling for each other and for externalizing symptoms ($P > 0.05$; **Table 3**). However, when their covariation was considered, Pearson-*r* partial correlations revealed that, for the total sample, adolescent amygdala-vmPFC rs-FC was significantly inversely correlated with adolescent anxiety and significantly positively correlated with adolescent depression ($P < 0.05$). When considered separately for males and females, results suggested, again, that these effects were largely specific to females. Significant associations ($P < 0.05$) between childhood cortisol and adolescent internalizing symptoms suggested that amygdala-vmPFC rs-FC might have a mediating role, especially for females.

We constructed a second moderated mediation SEM to examine whether adolescent amygdala-vmPFC rs-FC was a mediator in the association of childhood cortisol and adolescent anxiety symptoms, and whether this process was moderated by gender. This model (**Fig. 3**) demonstrated good fit and accounted for 64.6% of the variance in adolescent anxiety symptoms. There was no direct effect of childhood cortisol amount on adolescent anxiety symptoms, and the mediating effect of adolescent amygdala-vmPFC rs-FC was marginally significant (indirect path estimate = 0.13, $P < 0.10$). Gender significantly ($P < 0.05$) moderated the association of childhood cortisol level

Table 3 Pearson-*r* bivariate and partial correlation statistics for anxiety and depression

Average values of predictors	Anxiety			Depression		
	Females *r* value	Males *r* value	Total *r* value	Females *r* value	Males *r* value	Total *r* value
Correlations						
ELS	0.21	0.33#	0.30*	0.13	0.20	0.21
Childhood cortisol amount	0.35#	0.03	0.30*	0.07	–0.13	0.07
Adolescent amygdala-vmPFC rs-FC	–0.32#	0.18	–0.20	–0.001	0.14	0.001
Partial correlations						
ELS	0.20	0.22	0.22	0.02	–0.05	–0.03
Childhood cortisol amount	0.47*	0.13	0.40*	–0.35#	–0.21	–0.27*
Adolescent amygdala-vmPFC rs-FC	–0.56*	0.15	–0.35*	0.45*	0.05	0.27*

$n = 57$ (28 female). #$P < 0.10$, *$P < 0.05$. Partial correlations: to remove shared variance among the types of symptoms, depression and externalizing were selected from correlations with anxiety, and anxiety and externalizing were selected from the correlations with depression.

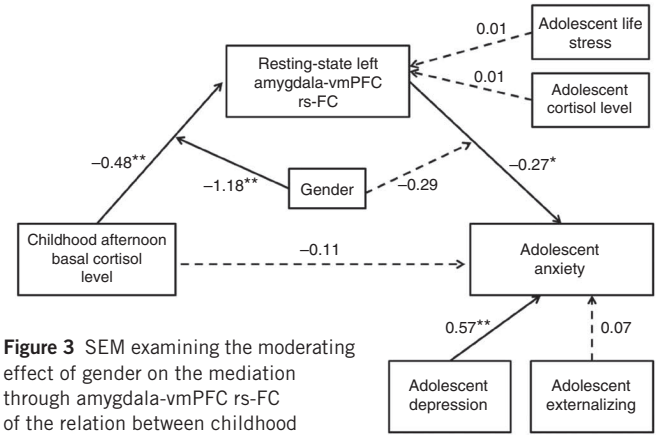

Figure 3 SEM examining the moderating effect of gender on the mediation through amygdala-vmPFC rs-FC of the relation between childhood late afternoon basal cortisol level and adolescent anxiety. The SEM demonstrated good fit: $\chi^2 = 9.95$, $P > 0.05$; root mean square error of approximation (RMSEA) = 0.11; standardized root mean square residual (SRMR) = 0.04; comparative fit index = 0.94. Paths are marked with unstandardized coefficients. $^*P < 0.05$, $^{**}P < 0.01$.

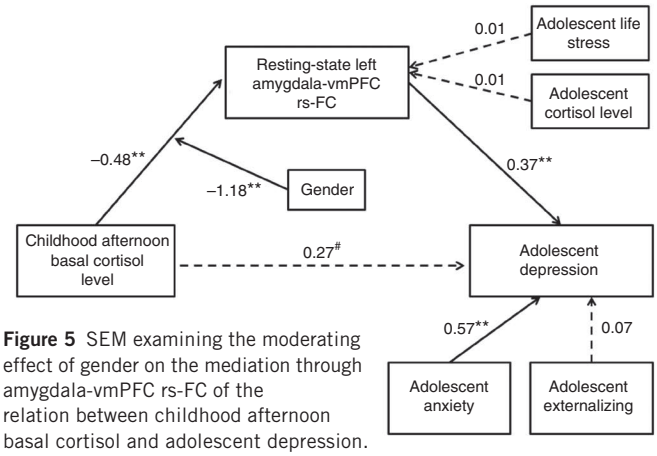

Figure 5 SEM examining the moderating effect of gender on the mediation through amygdala-vmPFC rs-FC of the relation between childhood afternoon basal cortisol and adolescent depression. The SEM demonstrated good fit: $\chi^2 = 7.56$, $P > 0.05$; root mean square error of approximation (RMSEA) = 0.07; standardized root mean square residual (SRMR) = 0.03; comparative fit index = 0.97. Paths are marked with unstandardized coefficients. $^{\#}P = 0.07$, $^*P < 0.05$, $^{**}P < 0.01$.

with rs-FC. However, because this model revealed a nonsignificant moderating effect of gender on the association of rs-FC with anxiety ($P = 0.23$), we constructed an alternate model without this pathway. In addition, because rs-FC estimates were obtained concurrently with anxiety symptoms, and there was no temporal precedence by which to define mediation, we built a second alternate model with the positions of anxiety symptoms and rs-FC reversed. For both of these models, the fit statistics were decreased and the model fit was poor (Online Methods). Together, these results show that, especially for females, amygdala-vmPFC rs-FC negatively correlated with concurrent symptoms of anxiety (**Fig. 4**). For females only, the developmental pathway between childhood cortisol level and adolescent anxiety symptoms operated through adolescent amygdala-vmPFC rs-FC.

To examine depression as an outcome, we built a parallel moderated mediational model. Although this model demonstrated good fit, gender did not moderate the path from amygdala-vmPFC rs-FC to depression and thus we omitted that pathway (Online Methods). This modified model demonstrated improved fit and accounted for 56.2% of the variance in depression (**Fig. 5**). There was a marginally significant ($P < 0.10$) direct effect of childhood cortisol on adolescent depressive symptoms, and the mediating effect of adolescent amygdala-vmPFC rs-FC was significant (indirect path estimate = −0.18, $P < 0.05$). As with anxiety, when depressive symptoms and amygdala-vmPFC rs-FC were transposed in an alternative model, the fit statistics were decreased, and the model fit was poor (Online Methods). Together, these results show that, for all adolescents, increased amygdala-vmPFC functional connectivity is associated with the development of depressive symptoms. In addition, for females only, the developmental pathway between childhood cortisol level and adolescent depressive symptoms operates, in part, through adolescent amygdala-vmPFC rs-FC.

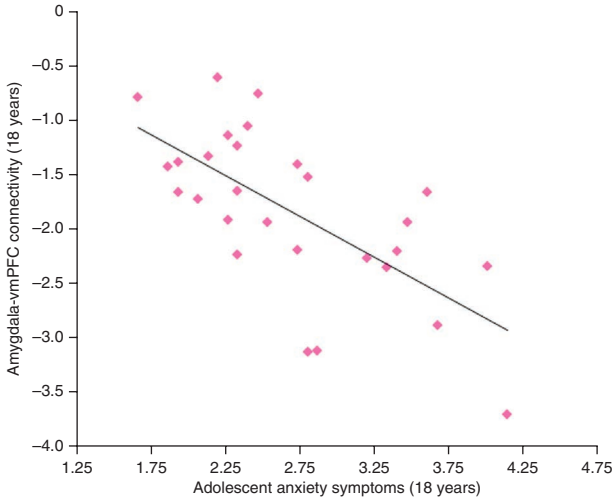

Figure 4 Partial correlation between resting-state left amygdala-vmPFC rs-FC and concurrent self-reported anxiety symptoms in adolescent females, controlling for concurrent symptoms of depression and externalizing behaviors ($R^2 = 0.31$, $P = 0.004$).

DISCUSSION

These findings establish a compelling developmental sequence whereby, in females, exposure to ELS during infancy was associated with higher levels of cortisol in childhood, and higher levels of cortisol at this early age predicted more negative amygdala-vmPFC rs-FC at age 18. Moreover, females with greater inverse adolescent amygdala-vmPFC rs-FC had higher levels of anxiety at 18 years, and subsequent analyses suggested that the developmental pathway from cortisol to anxiety worked through amygdala-vmPFC resting-state connectivity. Despite their temporal proximity, concurrent measures of adolescent life stress and cortisol were not significantly ($P < 0.05$) associated with amygdala-vmPFC rs-FC, implying that patterns of resting-state connectivity in adolescence likely reflect the cumulative impact of influences that begin early in life. Models examining anxiety controlled for depressive and externalizing symptoms, suggesting that this pathway is specific to anxious symptoms, adding to a growing literature that underscores the role of the amygdala-vmPFC circuit in anxiety[14–16].

Our findings on relations between amygdala-vmPFC rs-FC and depression stand in marked contrast to those for anxiety and they are consistent with recent findings that show increased connectivity between medial prefrontal and emotion circuits[26]. Such increased connectivity has been interpreted as reflecting emotionally charged self-focused rumination in depression. Whether there exist partially separable zones within vmPFC that may inhibit versus activate the

amygdala should be the focus of explicit investigation to better understand how the vmPFC may be playing these different roles in the etiology of anxious and depressive symptoms.

Our findings are to our knowledge the first to suggest a neurodevelopmental mechanism by which ELS increases vulnerability for internalizing disorders in adolescence. Our model indicates that ELS increases basal cortisol, which in turn modulates emotion-regulation circuitry in adolescence. The functional status of this circuitry at rest predicts both anxious and depressive symptoms and displays strong gender differences, with the effects being stronger in females. The fact that only females evinced these longitudinal associations between ELS during infancy, childhood late afternoon basal cortisol and neural connectivity in adolescence extends previous work suggesting that females may be more sensitive to the effects of ELS on neuroendocrine function[11] and work showing that females may also show more robust epigenetic changes in response to early social environmental factors[37].

These data provide new insight regarding the interplay among gender, ELS, HPA-axis function, resting-state brain activity and behavior. However, the causal relation among these constructs is not known. It is critical that future studies obtain measures of early childhood brain structure and function to determine whether connectivity differences are present early in life or, rather, are the product of developmental influences of ELS and early variation in HPA-axis function. Our understanding of these associations would also benefit from studies designed to examine relations among rs-FC, task-induced connectivity assessed with fMRI, and white matter microstructure with diffusion tensor imaging to probe for associations among these different indices of connectivity.

In conclusion, our findings imply that, in females, ELS and early cortisol function may leave an imprint on the brain that can be detected in adolescent resting-state connectivity within a circuit important for regulation of emotion, and variation in the function of this circuit is important in adolescent anxiety and depressive symptoms. The difference in direction of association between connectivity and anxious versus depressive symptoms is consistent with prior data. However, the factors that are responsible for these diverging patterns of association require additional study. It may be that among individuals predisposed to rumination, high levels of childhood cortisol sensitize the emotion-regulation circuitry in a way that results in greater emotional arousal to self-relevant rumination, resulting in a positive coupling between vmPFC and amygdala. Future research with systematic observations of stress, neuroendocrine function and brain activity throughout childhood and adolescence is needed to determine the causal role ELS may have in influencing HPA-axis function and resting-state connectivity in circuits that regulate emotion and to identify the factors that account for the pronounced gender difference we uncovered.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.A.B., D.E.S., P.L.R., J.M.A., M.J.E., R.J.D. and R.M.B. wrote and revised the manuscript. C.A.B., D.E.S., P.L.R., E.K.M., N.H.K., M.J.E. and R.M.B. performed data processing, statistical and/or image analyses. M.E.F. and A.S.H. collected data, created figures and assisted with editing the manuscript. C.A.B., D.E.S., P.L.R., J.A.O., M.J.E., R.J.D. and R.M.B. contributed to the interpretation of the data. M.J.E., R.J.D. and R.M.B. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Participants. Participants were 66 adolescents (32 female; mean age = 18.44, s.d. = 0.19 years) from the larger WSFW study[4], 57 (28 female) of whom had available childhood cortisol level data. Recruitment for the WSFW study began in 1990, and that study was designed to gather information on parental leave and health outcomes from a subsample of the general population in southern Wisconsin, USA. A total of 570 women and their partners were initially recruited from clinics and hospitals during routine prenatal visits. Mothers had to be over 18 years old, in their second trimester of pregnancy and living with their husband or partner. Selection for the present study was based on proximity to the laboratory and MRI exclusionary criteria. Participants' racial background was 53 Caucasian, 2 Native American/Alaskan and 2 African American. Data were collected during a 4-h laboratory visit. Informed consent (and parental permission in childhood) was obtained for all assessments, and participants received monetary compensation. University of Wisconsin-Madison Institutional Review Boards approved all procedures.

Measures of early life stress and childhood basal cortisol. ELS was indexed via a composite of maternal stress comprised of maternal reports of postnatal depression symptoms, marital conflict, parenting stress, financial stress and role overload as detailed elsewhere[4]. Scores from 1 month, 4 months and 12 months after birth were averaged to yield an infant maternal stress exposure measure.

Childhood basal cortisol (mean age = 4.58 years, s.d. = 0.05 years) was collected over 3 d. Parents were instructed to collect saliva samples at a target time between 3:00 p.m. and 7:00 p.m. In the current subsample, 84% complied with the prescribed time frame; nine had average collection times > 8 p.m. No differences in cortisol levels were detected as a function of time of collection ($r = 0.03$, $P = 0.90$), consistent with previous work demonstrating that evening cortisol is less likely to be impacted by small differences in collection time[19,38]. Children were allowed to be taking over-the-counter medications including nonsteroidal anti-inflammatory drugs (for example, ibuprofen) and/or allergy and cold medicine. The use of these medications was not associated with observed cortisol levels ($r = -0.11$, $P = 0.40$). Samples were assayed for cortisol level in duplicate using a radioimmunoassay modified for saliva (Pantex). The energy dispersive detection limit of the assay (ED_{80}) was 0.03 μg/dl, with mean inter- and intra-assay variations of 7.4% and 3.8%, respectively. Participants were included if they provided sufficient, uncontaminated samples on at least two of three days. Mean levels were normalized via $\log_{10}$ transformation and residualized for time of collection and use of over-the-counter medication[4].

Current cortisol, anxiety, depression and stress measures. Adolescent basal cortisol level collection followed similar procedures as in childhood, with participants collecting samples for 3 d at home near the date of the imaging visit. Average collection time in the current subsample was 5:21 pm (s.d. = 72 min). Samples were assayed in duplicate using a high-sensitivity salivary enzyme immunoassay kit (Salimetrics) with calibrator range of 0.012–3.000 μg/dl and mean intra- and inter-assay coefficients of variation of 3.5% and 5.1%, respectively. All other procedures mirrored those for the childhood cortisol level collection.

Adolescent psychiatric symptoms were assessed via self-report with the adolescent version of the MacArthur Health and Behavior Questionnaire[33] (HBQ), a well-validated measure of mental health, physical health, and social and academic functioning. HBQ subscales measuring symptoms of anxiety, depression and externalizing behaviors (for example, aggression, oppositional defiant disorder, conduct problems) were used in current analyses.

Lastly, current life stress was indexed using a 61-item life-events inventory modeled on the Adolescent Perceived Events Scale[34] and the Life Events Survey[35]. Events covered age-appropriate life domains (for example, relationships with friends/family, changes in parental marital status or finances, serious illnesses and deaths). The current analyses include the summed impact of negative events in the past six months.

Imaging data acquisition and processing. Structural and functional images were collected on a 3 T MRI scanner (Discovery MR750, General Electric Medical Systems) with an 8-channel radio-frequency (RF) head coil array. T1-weighted structural images (1 mm^3 voxels) were acquired axially with an isotropic MPRAGE sequence (echo time (TE) = 3.18 ms, repetition time (TR) = 8.13 ms, tip angle (TI) = 450, flip angle = 12 degrees). Participants were instructed to rest silently with their eyes closed while remaining "clear, calm and awake" during the collection of a T2*-weighted gradient-echo echo-planar pulse sequence lasting 420 s (210 volumes) with a TE, TR and flip angle of 25 ms, 2,000 ms and 60 degrees, respectively. Image volumes had a resolution of 3.5 mm × 3.5 mm × 5 mm (matrix size = 64 × 64; 30 sagittal slices).

Most data reduction steps were performed using AFNI software package[39]. Images were corrected for slice-dependent time shifts and motion and field-map corrected using FSL's PRELUDE[40] and in-house software. Anatomical images were aligned to the fifth volume of the echo-planar imaging (EPI) time-series using a Local Pearson Correlation cost function[41]. The first four volumes of the time-series were removed owing to T1-equillibrium effects. Images were then transformed to Talairach Atlas space[31] using a nine-parameter affine transformation and resampled to 2-mm cubic voxels.

Resting-state fMRI time-courses were temporally filtered (band-pass: 0.001 Hz < f < 0.01 Hz). To further reduce the influence of motion, time-points were censored if the motion of a point 87 mm from the center of rotation was >2 mm per degree. Variance from sources of non-interest was then regressed (AFNI's 3dDeconvolve function). Six rigid-body motion parameters were included as nuisance regressors, along with the voxel-wise average signal and derivatives from both eroded cerebral spinal fluid and 2× eroded white-matter masks. Masks were generated with an automated segmentation of the T1-weighted structural scan using FSL's FAST routine[40,42] and transformed to Talairach Atlas space[31]. EPI time series were spatially smoothed with a 6 mm full-width half-maximum (FWHM) Gaussian kernel (post-nuisance regression to avoid partial volume averaging within cerebral spinal fluid and white matter masks).

Statistical analyses. Rs-FC estimates were computed using a seed-region-based approach[43]. Binary masks of the left and right amygdala were defined in AFNI using previously developed boundaries[29]. The average preprocessed fMRI signal intensity time course over each amygdala region of interest was then regressed against the signal intensity time courses of all other voxels in the brain. Time points were motion-censored as outlined above. The correlation coefficient of each voxel in the resultant statistical parametric maps was converted into a Z-score using the Fisher Z-transformation. Participant connectivity maps were then entered into two-tailed regressions (AFNI's 3dttest++) while covarying behavioral variables of interest. Cluster sizes were selected based on α significance values less than or equal to 0.05 and were estimated with AFNI's 3dClustStim and 3dFWHMx.

In addition to the vmPFC, voxel-wise analyses with childhood evening cortisol revealed significant negative correlations with functional connectivity estimates between the left amygdala and clusters in the posterior cingulate and cerebellum (**Table 1**). Only the vmPFC-amygdala rs-FC remained significant when the two individuals with the highest cortisol values were completely excluded from analyses (before winsorization), $t(54) = -5.69$, $\beta = -0.62$, $R^2 = 0.37$, $P < 0.001$. To rule out the effects of subject motion, basal cortisol in adolescence and current life stress, each of these variables were entered into the previous models as covariates. None contributed significant variance to the observed relations between childhood cortisol and amygdala-vmPFC rs-FC, nor did they appreciably change these findings (individual β values ranged from −0.014 to −0.066, $P > 0.05$).

Next, to determine whether the maternal stress composite was the best predictor of the observed rs-FC, as mediated by childhood cortisol level, each maternal stress variable's association with childhood cortisol and rs-FC was examined independently. No significant associations were detected among the variables of interest in males. In females, only the financial stress subscale was marginally correlated with both childhood cortisol and significantly correlated with amygdala-vmPFC rs-FC (**Supplementary Table 1**).

Bivariate Pearson-r (two-tailed) correlations revealed significant comorbidity of anxiety and depression (females: $r = 0.74$, $P < 0.001$; males: $r = 0.68$, $P < 0.001$; total: $r = 0.76$, $P < 0.001$) as well as anxiety and externalizing (females: $r = 0.57$, $P < 0.005$; males: $r = 0.56$, $P < 0.005$; total: $r = 0.49$, $P < 0.001$), and depression and externalizing symptoms (females: $r = 0.51$, $P < 0.01$; males: $r = 0.47$, $P < 0.01$; total: $r = 0.45$, $P < 0.005$) in the current sample. To ensure specificity of symptom type, partial correlations were used which controlled for depression and externalizing problems when associations with anxiety were being examined, and anxiety and externalizing symptoms when associations with depression were being examined (**Table 3**).

Three primary SEMs were constructed to test anticipated mediating and moderating associations using the software package Mplus[36] (version 5.2). SEM provides estimates, or path coefficients, which indicate the direction and significance of the association between constructs, as well as several fit indices which evaluate the fit of the proposed model. A chi-squared significance test, considered good when nonsignificant, suggests the specified model is congruent with the observed data and is a reasonable measure of fit, particularly for models with small sample sizes[44]. The root mean square error of approximation (RMSEA) is considered adequate below 0.10, but may be used with caution as a fit index in small sample sizes. The standardized root mean square residual (SRMR) is the standardized difference between the observed correlation and the predicted correlation, and considered acceptable with values at 0.08 or less[45]. The comparative fit index (CFI) considers the number of parameters, or paths, in the model and is considered good at 0.93 or above. Whereas ideally models would have a sample size to number of free parameters ratio of 20:1, a more realistic ratio of 5:1 is acceptable[46]. To test for mediation in all three models, both direct and indirect effects (path estimates) were examined. To test for moderation, main and appropriate interactive effects of sex were included on all indirect pathways[47].

The first model examined whether childhood cortisol mediated the association between ELS exposure and amygdala-vmPFC rs-FC and tested to see if both portions of the indirect pathway were moderated by gender. Additionally, adolescent current life stress and basal afternoon cortisol were included as predictors of rs-FC to ascertain whether the observed effects were indeed specific to ELS and/or childhood basal cortisol function. The second model tested whether adolescent amygdala-vmPFC rs-FC was a mediator in the association of childhood cortisol and adolescent anxiety symptoms. Again adolescent current life stress and basal cortisol were included as predictors as well as depression and externalizing symptoms. This model (**Fig. 3**) accounted for 64.6% of the variance in adolescent anxiety symptoms, and demonstrated a marginally-significant mediating effect of adolescent amygdala-vmPFC rs-FC (indirect path estimate = 0.13, $P < 0.10$). While this model revealed a nonsignificant moderating effect of gender on the association of rs-FC with anxiety ($P = 0.23$), removing this pathway resulted in poor model fit ($\chi^2 = 17.15$, $P < 0.05$, RMSEA = 0.19, CFI = 0.84). Finally, because rs-FC were obtained concurrently with anxious symptoms an additional SEM was constructed with the positions of anxiety symptoms and rs-FC reversed. The fit statistics of this model were poor ($\chi^2 = 17.09$, $P < 0.05$, RMSEA = 0.19, CFI = 0.84), and there was no mediational effect (indirect path estimate = –0.01, $P > 0.05$).

A third model, parallel to the model for adolescence anxiety, tested the same associations in relation to adolescent symptoms of depression. This model demonstrated good fit ($\chi^2 = 9.67$, $P > 0.05$, RMSEA = 0.11, CFI = 0.94), but gender did not moderate the path from rs-FC to depression ($P > 0.05$). When that pathway was omitted, the final model demonstrated improved fit ($\chi^2 = 7.56$, $P > 0.05$, RMSEA = 0.07, CFI = 0.97) and accounted for 56.2% of the variance in depression (**Fig. 5**). As with anxiety, when depressive symptoms and amygdala-vmPFC data were transposed in a subsequent model, the fit statistics were reduced to unacceptable levels ($\chi^2 = 25.44$, $P < 0.05$, RMSEA = 0.25, CFI = 0.66) and there was no mediational effect (indirect path estimate = 0.02, $P > 0.05$).

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