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Amygdala reward reactivity mediates the association between preschool stress response and depression severity

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Abstract

Background: Research in adolescents and adults has suggested that altered neural processing of reward following early life adversity is a highly promising depressive intermediate phenotype. However, very little is known about how stress reactivity, neural processing of reward, and depression are related in very young children. Motivated by this knowledge gap, the present study examined the concurrent associations between cortisol response following a stressor, functional brain activity to reward, and depression severity in 4-6 year old children.

Methods: Fifty-two medication naïve 4-6 year olds participated in a study using functional magnetic resonance imaging (fMRI) to assess neural reactivity to reward, including gain, loss, and neutral outcomes. Parent-reported child depression severity and child cortisol response following stress were also measured.

Results: Greater caudate and medial prefrontal cortex reactivity to gain outcomes and increased amygdala reactivity to salient (i.e., both gain and loss) outcomes were observed. Higher total cortisol output following a stressor was associated with increased depression severity and reduced amygdala reactivity to salient outcomes. Amygdala reactivity was also inversely associated with depression severity and found to mediate the relationship between cortisol output and depression severity.

Conclusions: Results suggest that altered neural processing of reward is already related to increased cortisol output and depression severity in preschoolers. They also demonstrate an important role for amygdala function as a mediator of this relationship at a very early age. Our results further underscore early childhood as an important developmental period for understanding the neurobiological correlates of early stress and increased risk for depression.
INTRODUCTION

Major depressive disorder (MDD) is one of the most common psychiatric conditions and a leading cause of impairment, disability, and morbidity (1). Given a growing consensus that the origins of depression are likely neurodevelopmental in nature (2), remarkably little is known about its neurobiological roots. As a result, identifying early occurring neurobiological intermediate phenotypes associated with depression is critical for advancing efforts to establish predictive biomarkers of relative risk and resilience to this disorder. Research now clearly demonstrates that depression during the preschool period is a precursor of later school age and adolescent MDD (3, 4). As such, investigations of brain function in preschoolers with elevated symptoms of depression are likely to provide crucial information informing the next generation of intervention strategies aimed at reducing the considerable public health burden of this disorder.

Altered neural processing of reward has emerged as a highly promising depressive intermediate phenotype (5). Reward processing relies on an interconnected network of brain regions, including the midbrain, amygdala, striatum, anterior cingulate cortex, orbitofrontal cortex, and medial prefrontal cortex (6). Functional magnetic resonance imaging (fMRI) research has provided key data supporting altered reward-related brain function in adults and adolescents with depression, including associations with depression severity (7), diminished daily experience of positive emotion (8), response to depression treatment (9), and later depression in adolescents (10, 11). Given that neural processing of reward undergoes a prolonged period of development beginning in early childhood (12), early experiences influencing this developmental process have been proposed to underlie the future emergence of depression in at least some individuals (13).
The very early experience of stress has emerged as one of the most salient factors that may negatively influence reward-related brain function and contribute to the development of depression (14). Consistent with this notion, recent research has shown that variability in neural response to reward partially mediates the relationship between stressful childhood experiences and elevated depressive symptomatology during adolescence and adulthood (15-17). However, this research has primarily relied on retrospective measures of early life stress and assessed brain function during adolescence or adulthood. As a result, whether similar associations are present in young children is unknown and the putative mechanisms through which early life adversity is associated with neural processing of reward remains poorly understood.

Emerging independent lines of evidence raise the possibility that hypothalamic–pituitary–adrenal axis (HPA) function may play a mechanistic role in the expression of early life stress-related neural reward processing dysfunction (14). First, preclinical work indicates that the development of reward-related brain regions rich in glucocorticoid receptors is negatively affected by increased levels of glucocorticoids during prolonged periods of elevated stress (18). Second, previous research has reported altered HPA reactivity in groups of children exposed to early stressful life events (19, 20) and attenuated reward-related brain function in adolescents and adults with a history of early life stress (17, 21), including those who eventually develop depression (15). Lastly, recent fMRI data suggests that acute cortisol administration blunts reward-related neural activity (22, 23). Collectively, these data suggest that altered HPA stress reactivity following repeated exposure to stressors during early childhood may result in relatively blunted neural responses to reward, potentially conferring increased risk for depression. However, data directly informing the relationship between HPA function and neural response to
reward during early childhood is not available. Such data would provide critical insight into our mechanistic understanding of how early life stress conveys increased risk for depression.

The present study investigates whether altered HPA functioning is associated with altered neural reactivity to reward and depression severity in preschoolers using fMRI. It also tests whether altered neural reactivity to reward mediates the relationship between cortisol output following stress and depression severity in preschoolers. Following previous research, it was predicted that greater depression severity in preschoolers would be linked to higher total cortisol output to an in-lab psychosocial stressor (24). Based on evidence that cortisol administration blunts reward-related activity in the amygdala and striatum, and data suggesting these regions as highly susceptible to the effects of early life stress and altered in pediatric depression (25) (26), we predicted that higher total cortisol output following stress would be associated with diminished reactivity to reward related outcomes in these regions. Lastly, we anticipated that altered neural reactivity to reward in these regions would mediate the relationship between cortisol output and depression severity.

METHODS AND MATERIALS

Participants:

Eighty-eight preschoolers between 4-6 years of age were recruited from pediatrician’s offices, daycares, and other community resources throughout the greater St. Louis area. In order to increase sample variance in depressive symptoms, a validated screening checklist (Preschool Feelings Checklist (27); PFC) was used to identify preschoolers with and without elevated depressive symptoms. Caregivers indicating that their preschoolers had “low” (≤1 PFC items endorsed) or “high” (≥3 PFC items endorsed) levels of depressive symptoms were contacted and invited to complete additional phone screening steps assessing for the presence of neurological
disorders (e.g., seizure disorder), autism spectrum disorders or developmental delays, premature birth (<36 weeks gestation), and psychotropic medication use. Endorsement of any of these conditions acted as exclusionary for all children. Children passing the exclusion criteria were invited to enroll in the full study. Following study enrollment, each family was asked to complete an age appropriate mental health and developmental assessment and an fMRI scan within 7-10 days of their assessment. Of the 88 children completing the study, complete fMRI data were not collected for 9 children due to equipment failure (N=3), falling asleep during scan (N=1), refusal to play fMRI task (N=1), or request to end scan (N=4). Of the 79 children completing the fMRI scan, 60 provided data passing quality control (QC) measures (76%; see Supplemental Information). Of the 60 children with usable fMRI data, 52 also had stress reactivity cortisol data passing QC (see Supplemental Information) and were included in the analyses addressing our a priori hypotheses. Parental written consent and child verbal assent were obtained for all subjects. The Institutional Review Board at Washington University in St. Louis approved all experimental procedures.

Diagnostic Assessment:

Diagnostic assessments were conducted using the Kiddie Schedule for Affective Disorders-Early Childhood version (K-SADS-EC; (28), a developmentally modified version of the Kiddie Schedule for Affective Disorders and Schizophrenia for School age Children-Present and Lifetime Version (K-SADS-PL)(29). See Supplemental Information for greater detail.

Depression Severity:

Child: The Preschool Feelings Checklist – Scale Version (PFC-S; (30) is a 23 item measure that uses a Likert rating scale (0 = never, 4 = most of time; range of possible scores 0-92) designed to assess depression severity in preschool children and has established validity at this age (31).
Example items include, ‘My child appears sad or says he/she feels sad’ and ‘Enjoys activities and play (reverse scored).’ See Supplemental Information for additional information.

Parent: Parents filled out the Beck Depression Inventory–II (BDI–II; (32), a validated 21-item measure of depression symptom presence and severity in adults.

Cortisol Collection and Analysis Procedures:

Children completed a stress-inducing ‘frustration’ task that reliably induces a cortisol response in preschoolers (33). Briefly, children were instructed to match colored wooden chips with corresponding shapes to earn a prize before time ran out (~3 minutes). A toy traffic light indicated how much time they had remaining and experimental manipulation of timing ensured task failure. One saliva sample was collected prior to the frustration task as a baseline measurement of cortisol (preceded by a half-hour period of neutral activities) and six saliva samples were collected every ten minutes during the hour following the task while a neutral movie was watched. See Supplemental Information for detailed collection, assay, and data quality control methods.

Consistent with prior observations, cortisol data were skewed and subsequently log_{10} transformed prior to all analyses (34). Following previous research suggesting that total cortisol output following stress is associated with depression and depression risk (20, 35), total cortisol production during the stress task was calculated using standard area under the curve with respect to ground (AUC_{g}) procedures (36), incorporating actual time between cortisol sample collection in these calculations.

Child fMRI Gambling Task:

fMRI data were collected as children completed the Child Gambling Task (CGT) approximately 7-10 days following their in-person assessment. The CGT is a developmentally adapted form of
a commonly used ‘gambling’ reward processing task (37) previously shown to elicit robust and reliable activation in reward related regions in older age groups (37-42). It has also been used in prior studies of reward and loss sensitivity in relation to depression (8, 15-17, 43-45). The CGT was presented with E-Prime (Psychology Software Tools, Inc.) using an event related design with 13 trials of each outcome (i.e., gain, loss, neutral) presented in a predetermined pseudo randomized order (no more than 3 of the same type in a row) per run (Figure 1). During the CGT, children are asked to guess whether the next person they see is going to be bigger or smaller than them to win or lose candy. To reduce the potential for movement, only one response (i.e., either ‘bigger’ or ‘smaller’) is assigned to a single button, with nonresponses (i.e., no button press) representing the alternate choice. The assignment of bigger or smaller as the active response was counterbalanced across children. The gain and loss amounts were chosen to give gains and losses of similar subjective values (46). Each child completed two ~6 minute runs and were given an amount of candy matching the maximum gained during the CGT following scan completion.

**Functional Imaging Data Acquisition and Preprocessing Procedures:**

To create familiarity and comfort with study procedures, each child was shown a child friendly video introducing the fMRI experience and introduced to the scanning environment using a mock scanner training protocol during their initial in-person assessment, allowed to watch a movie of their choice during structural scans, and rewarded with small prizes following scan completion. Imaging data were collected using a 3T TIM TRIO Siemens whole body system. See **Supplemental Information** for fMRI acquisition and preprocessing procedures.

**Functional Imaging Data Analysis:**

A general linear model (GLM) approach incorporating regressors for outcome, linear trend, and
baseline shift was used to estimate subject-specific voxel-wise task-related activity without assuming a hemodynamic response shape. Gain, loss, and neutral outcomes were modeled separately relative to fixation baseline for 10 frames following question mark onset (Figure 1). The estimates for the last 8 frames represent the different time points in 2-second increments following presentation of the reward outcome. The resulting beta estimates of the event-related response at each frame were entered into a second-level analysis treating subjects as a random factor. At the second level, we computed a voxel-wise repeated-measures analysis of variance (ANOVA) with time point (10 estimated frames) as a within-subject factor.

Both region-of-interest (ROI) and whole-brain approaches were used. The more conservative ROI approach was conducted using two a priori masks focused on 1) the left and right amygdala adopted from (47) and 2) an a priori network of regions implicated in reward processing including the dorsal and ventral striatum adopted from (42, 48). The choice of these two ROIs was based upon evidence indicating 1) that the amygdala plays an important and specific role in evaluating reward salience (49, 50), 2) amygdala reactivity is altered in depressed preschoolers (51), and 3) that developmental and depression related differences in striatal and cortical response to reward can be successfully identified in children using our a priori mask of reward-related regions (41, 52). To isolate task-evoked amygdala signals, we initially computed our ANOVA using the individually averaged beta values for each time point from our a priori amygdala ROI. Subsequent ANOVAs using our a priori reward processing mask or at the whole brain level were corrected for multiple comparisons (see Supplemental Information for additional details).

Following the identification of a significant main effect of time within a given brain region (e.g., amygdala), timecourses were subsequently inspected for time x outcome interactions using
a 2-way repeated-measures ANOVA. When an outcome x time interaction was identified for a given brain region, follow-up paired t-tests were used to identify at which time point(s) conditions differed. Following previous event-related fMRI research (53-55), the two time points representing the period of peak difference between outcomes were identified, averaged within a given outcome (e.g., gain), and then subsequently subtracted between the differing outcomes (e.g., gain minus loss) to create a peak difference score. Peak difference scores were then examined in separate correlational and mediation analyses using PFC-S and AUC<sub>g</sub> cortisol scores and a 2-tailed approach to significance (IBM SPSS Statistics version 21; SPSS Inc., Chicago, IL, USA).

**Brain Function, Stress, and Depression Severity:**
In order to test our a priori hypothesis that attenuated neural response to reward mediates the relationship between altered HPA function and depression severity in preschoolers, we used the PROCESS macro procedure for SPSS. Following Hayes (56), a significant effect of mediation would indicate that the association between AUC<sub>g</sub> and depression severity occurs indirectly through brain activity. Only difference scores generated from our a priori ROIs with a time x outcome effect were examined in the mediation analyses (see Figure 2A for complete model). A multivariate approach to identifying potential outliers using Mahalanobis D<sup>2</sup> was conducted prior to carrying out our a priori correlational and mediation analyses. No outliers were identified.

**RESULTS**

**Demographic and Child Characteristics:**
See Table 1 for sample demographic and diagnostic characteristics. Averages scores were 16.1 (±6.3; range 1-47) for PFC-S, 8 (±9.2; range 0-34) for BDI-II, and 35.6 (±5.5; range 27.23-53.52) ng/ml for AUC<sub>g</sub>. Preschoolers with a diagnosis of MDD on the K-SADS-EC had higher
PFC-S scores that those who did not (MDD = 28 (±10), No MDD (12.5 (±8.4); t_{50} = 5.4, p < .001) and those not providing usable fMRI data were younger (mean age 60 [11.5] months) than those who did (mean age 71 [9] months). Previous research suggests that maternal mood state likely inflates parent report of child psychopathology. In line with this, there was a significant positive correlation between PFC-S and BDI-II scores (r = .56, p < .001) in the current sample. Thus, all analyses including the PFC-S controlled for maternal BDI-II scores.

Behavioral Results for Scanner Task:
On average, children pressed the response button on 56% (44/78) of the CGT trials. Reaction time (RT) was missing for two children who did not push the response button during the CGT. Average win RT = 1001ms (±219), average loss RT = 972ms (±208), and average neutral RT = 963ms (±215). RT did not differ between outcome conditions (all t_{[50]} ≤ 1.41, p ≥ .165).

Neuroimaging Findings:
A main effect of time was found for the left and right amygdala ROIs as well as for multiple regions within our a priori reward processing mask, including the left anterior insula, anterior cingulate cortex (ACC), and bilateral caudate (Table 2). Time x outcome interactions were also noted, including greater left and right caudate reactivity for gain versus loss outcomes, greater ACC reactivity for gain versus loss and neutral outcomes, and increased left amygdala reactivity following gain and loss outcomes versus neutral ones (Figure 3). Consistent with previous research suggesting the amygdala is sensitive to stimulus salience rather than valence (49, 57), our paired t-tests revealed that gain and loss timecourses in the left amygdala did not differ from each other and were identical in their pattern of peak differences with neutral outcomes. Thus, we used an averaged timecourse for gain and loss outcomes (gain/loss) when creating left amygdala difference scores. Follow-up paired t-tests identified time points five and six as the
period of peak difference between gain/loss and neutral outcomes in the left amygdala and between gain and loss and gain and neutral outcomes in the ACC. For the left and right caudate, follow-up paired t-tests indicated that peak differences between gain and loss outcomes were present at timepoints four and five. Individual peak difference scores were generated for the amygdala, caudate, and ACC (e.g., [average of gain timepoints 4 and 5] – [average of loss timepoints 4 and 5] for the left caudate) and used in all subsequent analyses.

Whole brain results were significant for a main effect of time in multiple cortical and subcortical regions. Follow-up analyses found outcome x time effects in parahippocampla gyrus, fusiform gyrus and postcentral gyrus. See Supplemental Information for additional information.

Brain Function, Stress, and Depression Severity:
Following our a priori hypotheses, AUC\(g\) was positively correlated with child depression severity (\(r = .32, p = .021\)) and negatively correlated with differences between gain/loss and neutral outcomes in the left amygdala (\(r = -.37, p = .006\)). In addition, differences between gain/loss and neutral outcomes in the left amygdala were negatively correlated with child depression severity (\(r = -.40, p = .003\); Figure 2B). Further, reduced gain/loss versus neutral difference scores in the left amygdala were found to mediate the significant relationship between elevated AUC\(g\) and increased depression severity in preschoolers (PROCESS Indirect Effect [10,000 bootstrap samples]: .2 (.11), bias corrected 95% CI: .05/.5, Figure 2A). The relationships between AUC\(g\) and left and right caudate gain versus loss difference scores were not significant, though in the expected direction (right caudate \(r = -.19, p = .17\); left caudate \(r = -.27, p = .052\)). AUC\(g\) was not related to either of the ACC difference scores (gain versus loss \(r = -.12, p = .39\); gain versus neutral \(r = -.22, p = .13\)). The pattern and significance of observed results did not
change when gender or age was included as a covariate. Please see Supplemental Information for additional analyses supporting the specificity of the mediation results to AUCg, neural response to highly salient (i.e., gain/loss) outcomes, and their robustness to additional covariates.

**DISCUSSION**

The current study used fMRI to examine whether neural reactivity to reward mediates the relationship between cortisol response following a stressor and depression severity in preschool age children. Our results extend prior reports in older age groups (14) by showing that both higher total cortisol output following a stressor and attenuated neural sensitivity to highly salient outcomes (i.e., gain and loss) are already related to increased depression severity in preschoolers. They also match prior findings suggesting a negative relationship between cortisol and reward-related brain activity (22, 23). Importantly, the current findings provide novel evidence further supporting attenuated neural sensitivity to reward-related information as a putative mechanism through which early life adversity is associated with increased risk for depression.

Attenuated neural processing of reward following early life stress has emerged as one of the most promising depressive intermediate phenotypes. More specifically, it has been suggested that under conditions of chronic stress and adversity, physiological responses to stress occur more frequently, tend to increase in magnitude and duration, and take longer to recover to baseline levels (58). Over time, the repeated, excessive activations and inefficient down-regulation of stress response systems - including the HPA – has a significant and negative affect on developing reward-related brain function, increasing risk for later MDD (59). However, data directly informing the relationship between individual HPA stress response and neural processing of reward during early childhood has remained largely unavailable, leaving the developmental trajectory of this intermediate phenotype uncharted. As a first step in filling this
knowledge gap, the current findings indicate that higher total cortisol output following a mild stressor in preschoolers is associated with diminished amygdala reactivity to highly salient reward processing outcomes. The amygdala has been consistently shown to play an important role in evaluating the motivational significance of a given stimulus (57). Recent work has suggested that stress may dampen amygdala reactivity in this regard. More specifically, oral administration of cortisol has been reported to dampen amygdala reactivity to reward in older samples (22, 23). Preclinical work has also suggested that chronic stress induces significant dendritic spine loss in the medial amygdala (60), a major efferent nucleus of the amygdala sensitive to the motivational salience of events and strongly interconnected with the mesolimbic dopamine pathway (61). The current findings extend this work by providing unique insight into how developing stress and brain reward systems are related to each other very early in life. They also provide critical support for theoretical models suggesting that repeated activation of the HPA system may eventually facilitate the development of attenuated neural reactivity to reward as a more stable ‘trait’ like neurobiological endophenotype linking early adversity and MDD risk (14) (62). However, the current findings cannot address to what degree attenuated amygdala reactivity in our preschoolers is reflective of repeated exposure to prolonged HPA stress-related activity or establish a causal relationship. Nevertheless, they do provide important evidence suggesting that stress and brain reward systems are already tightly entwined as early as the preschool period.

Disrupted incentive-based learning has emerged as one potential mechanistic explanation of how altered reward processing mediates the relationship between early stress and increased risk for depression (14). Appropriate processing of reward outcomes plays a central role in incentive-based learning, with intact sensitivity to salient events (e.g., gains and/or losses)
believed to be critical for learning reward-predicting cues that shape later self-regulation and
goal directed behavior (63), both of which are disrupted in depression. Behavioral studies
indicate that developmental changes in reward learning are already underway during the
preschool period (64-66). Importantly, this work also suggests that developmental changes in
early reward learning may lay a critical foundation for the ongoing development of self-
regulation (64) and goal directed behavior (66). For example, recent behavioral data has
illustrated that intact sensitivity to gain outcomes results in increased inhibitory control in
preschoolers (66) and that diminished reward learning is associated with significant behavior
regulation difficulties at this age (64). Previous work has suggested that the amygdala plays a
critical role in reward learning; with disruptions affecting the ability to acquire as well as
generalize learned responses (49, 50). Previous preclinical work also suggests that early
disruptions in amygdala functioning may negatively influence the ongoing development of later
maturing brain regions also important for reward processing and learning, including the medial
prefrontal cortex (67). However, longitudinal studies beginning very early in development will
be needed to more fully understand the complex relationships between brain development,
reward learning, and emerging depression.

In contrast to previous work, higher total cortisol output following a stressor was not
associated with caudate reactivity to gain versus loss. Previous research has suggested that
attenuated reward-related activity in the striatum may be most evident during the experience of
an acute stressor (23, 68, 69). Given the very young age of our children, cortisol response to
stress was measured prior to their scan. As a result, the current study is unable to inform the
relationship between cortisol and caudate reactivity when measured concurrently. Interestingly,
recent functional connectivity work has suggested that the amygdala and striatum are positively
connected in preschoolers, adolescents, and adults (70). As a result, it has been speculated that early alterations in amygdala reactivity to stimulus salience may negatively influence ongoing development of the striatum, with altered striatal response to reward following early stress emerging later in development as a result (25). However, longitudinal studies will be needed to answer this question. Alternatively, stress related attenuation of reward processing in the caudate might be most apparent during tasks involving reward anticipation and/or learning (71), two aspects of reward processing not directly tested in this study. Future work directly investigating these possibilities will be necessary to better understand the relationship between stress and caudate activity during early childhood.

Several limitations should be noted. First, future investigations into other constructs (e.g., threat processing) and disorders (e.g., anxiety) will be necessary to inform the specificity of our results to reward processing and depression. Given that all measures were taken concurrently, the current results cannot inform directions of causality (see Supplemental Information for discussion of alternative mediation models). As a result, longitudinal studies will likely be critical for identifying trajectories of risk for depression and related psychopathology and informing interventions that can successfully target them. Nevertheless, the current study supports stress attenuated neural sensitivity to salient, reward-related outcomes as one potential mechanism that increases depression risk and further underscores early childhood as an important developmental period for understanding its earliest roots (72).
ACKNOWLEDGEMENTS

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FINANCIAL DISCLOSURE

The authors report no biomedical financial interests or potential conflicts of interest.
REFERENCES


Figure 1: Child Gambling Task (CGT). Each trial of the CGT begins with a white fixation cue presented in the center of a black screen for 2000ms. Next, a screen displays a question mark for 2000ms. Children are asked to guess whether the person hiding behind the question mark is bigger or smaller than them and to indicate their choice by pressing a button on an MRI compatible single button response box designed specifically for use with young children. Following their choice, feedback is generated as a function of whether the trial was scheduled to be a reward, loss or neutral outcome and presented for 2000ms. Feedback images included either a baby, adult, or similarly sized child paired with: 1) a green up thumbs up next to 4 candies for gain, 2) a red thumbs down next to an image of 2 candies with a line through them for loss; or 3) two dashes (“- -“) for neutral trials. A jittered inter-trial interval using a black screen with central fixation cross occurred between each trial (M=4000ms, Min.=2000ms, Max=6000ms).

Figure 2: A) Attenuated differential responding in the left amygdala to gain/loss versus neutral outcomes mediates the relationship between elevated stress reactivity and depression severity in preschool age children. Values represent beta coefficients generated by the SPSS PROCESS macro procedure for mediation model 4. a = p < .05; b = includes maternal depression as a covariate; c = includes maternal depression and stress reactivity as covariates B) Scatter plots illustrating the positive correlation between AUCg and child depression severity (r = .32, p = .021), the negative correlation between gain/loss minus neutral difference scores in the left amygdala and AUCg (r = -.37, p = .006), and the negative correlation between gain/loss minus neutral difference scores in the left amygdala and child depression severity (r = -.40, p = .003). Plots including depression severity scores represent the residualized values for each variable after controlling for maternal depression.

Figure 3: Differential responses to reward outcomes were found in bilateral caudate and left amygdala. Specifically, greater reactivity to gain versus loss outcomes was found in the left and right caudate while great reactivity to both gain and loss outcomes versus neutral ones was found in the left amygdala. Dashed boxes highlight frames used to generate difference scores. ? = task guess period; OC = task outcome period
Table 1: Study Group Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N = 52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>71.9 (±8.9)</td>
</tr>
<tr>
<td>Gender</td>
<td>28F/24M</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>35W/14AA/3O</td>
</tr>
<tr>
<td>PFC Screen(^a)</td>
<td>34 low/18 high</td>
</tr>
<tr>
<td>Diagnoses(^b)</td>
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<tr>
<td></td>
<td>Internalizing 9</td>
</tr>
<tr>
<td></td>
<td>Externalizing 2</td>
</tr>
<tr>
<td></td>
<td>Int. and Ext. 4</td>
</tr>
</tbody>
</table>

Note. F = female; M = male; W = white; AA = African American; O = other; PFC = Preschool Feelings Checklist

\(^a\)Number of children with caregiver reporting “low” (\(\leq 1\) PFC items endorsed) or “high” (\(\geq 3\) PFC items endorsed) levels of depressive symptoms during initial screen

\(^b\)Internalizing: Preschool Depression (N=8), Preschool Depression and Separation Anxiety Disorder (N=1), Generalized Anxiety Disorder (N=1)

Externalizing: Oppositional Defiant Disorder (N=1), Attention-Deficit Hyperactivity Disorder (N=1)

Internalizing and Externalizing: Preschool Depression and Oppositional Defiant Disorder (N=2), Oppositional Defiant Disorder and Attention-Deficit Hyperactivity Disorder (N=1)
Table 2: Regions identified in a priori reward mask with main effect of time.

<table>
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<th>Region</th>
<th>Hemisphere</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>Cluster (voxels)</th>
<th>Outcome X Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globus Pallidus (includes amygdala)</td>
<td>R</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>63</td>
<td>NS</td>
</tr>
<tr>
<td>Caudate</td>
<td>L</td>
<td>-10</td>
<td>3</td>
<td>3</td>
<td>205</td>
<td>NS</td>
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<tr>
<td>Caudate*</td>
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<td>-10</td>
<td>-4</td>
<td>18</td>
<td>34</td>
<td>G &gt; L</td>
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<td>6</td>
<td>4</td>
<td>32</td>
<td>NS</td>
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<tr>
<td>Medial Globus Pallidus* (includes amygdala)</td>
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<td>-12</td>
<td>0</td>
<td>-5</td>
<td>35</td>
<td>NS</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>R</td>
<td>10</td>
<td>-21</td>
<td>-9</td>
<td>42</td>
<td>NS</td>
</tr>
<tr>
<td>Red Nucleus</td>
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<td>-21</td>
<td>-6</td>
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<td>21</td>
<td>22</td>
<td>G &gt; L, N</td>
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</tbody>
</table>

*Following application of peak splitting algorithm to caudate cluster. BA = Brodmann Area; G = gain; L = loss; N = neutral; NS = not significant
A

-0.07 (.03)

Stress Response

Depression Severity

Left Amygdala

-6.6 (2.9)

.34 (.23), p = .15

.54 (.22), p = .02

Direct effect prior to mediation

Direct effect after mediation

B

Area Under the Curve - Cortisol

Depression Severity

Left Amygdala

Partia Regression Plot
Amygdala Reward Reactivity Mediates the Association Between Preschool Stress Response and Depression Severity

Supplemental Information

Diagnostic Assessment

Diagnostic assessments were conducted using the Kiddie Schedule for Affective Disorders-Early Childhood version (K-SADS-EC; (1)), a developmentally modified version of the Kiddie Schedule for Affective Disorders and Schizophrenia for School age Children-Present and Lifetime Version (K-SADS-PL) (2) adapted for use in preschool age children. Revisions include adjusting and/or adding questions and threshold anchors reflecting developmentally appropriate symptom and diagnostic criteria for 3-6 year old children, removing sections less applicable to young children, editing probes to use parent-directed wording, and providing directions for clarifying symptom severity and genesis where necessary. Previous research has demonstrated diagnostic consistency between the K-SADS-PL and the Preschool Age Psychiatric Assessment (3), a valid and commonly used semi-structured psychiatric interview for parents of young children. Following completion of the K-SADS-EC by a trained research assistant, relevant symptom and duration criteria gathered during the interview were individually reviewed for each child by a licensed clinical psychologist with expertise in early childhood development and psychopathology (author MSG) and subsequently used to generate DSM-5 (4) diagnoses when appropriate.

Depression Severity

The Preschool Feelings Checklist – Scale Version (PFC-S; (5) is a 23 item measure that uses a Likert rating scale (0 = never, 4 = most of time; range of possible scores 0-92) designed to assess depression severity in preschool children and has established validity at this age (6). To further
support the validity of the Preschool Feelings Checklist – Scale version (PFC-S) as a measure of
depression severity and to demonstrate its convergence with a widely available clinical
questionnaire commonly used to examine emotion difficulties in preschoolers, we examined the
relationship between scores from the PFC-S and the Child Behavior Checklist (CBCL) (7) subscale
scores for Depression/Anxiety and Affective Problems (reflecting many of the symptoms of
depression including sadness, irritability, diminished positive affect, sleep disturbances, altered
eating, and fatigue in the current sample). CBCL scores were available for 50 of the 52
preschoolers included in the current study. As anticipated, the PFC-S and both CBCL scores were
highly positively correlated (PFC-S and CBCL Depression/Anxiety raw score: r = .55, p < .001;
PFC-S and CBCL depression/anxiety t-score: r = .53, p < .001; PFC-S and CBCL Affective
Problems raw score: r = .73, p < .001; PFC-S and CBCL Affective Problems t-score: r = .72, p <
.001), providing further support of the PFC-S as a valid measure of depression severity and its use
as such in the current study.”

Cortisol Assay and Quality Control Methods

Saliva samples (n=395) were collected using Salivette collection devices (Sarstedt, Rommelsdorf,
Germany) then stored at -80°C immediately following collection. Salivary cortisol was measured
using enzyme-linked immunosorbent assay (ELISA; DRG International kit SLV-2930 lot #s
64K035 and 64K105; Springfield, New Jersey USA). All samples were run in duplicate and the
average value was used for analyses. Intra- and inter-assay coefficients of variation (CV) were
3.99% and 6.85% respectively.

Prior to use, kits, reagents, and samples were brought to room temperature. Samples were
centrifuged at 3,000 x g for 10 minutes. Next, 120 µL of sample, standard (0.0, 2.0, 5.0, 10.0, 20.0,
40.0, 80.0 ng/mL), or high and low cortisol control samples (to allow for interplate comparison) were aliquoted to a clean 96 well plate. Then, 100 µL from each well was transferred to the 96 well ELISA plate pre-coated with mouse anti-cortisol antiserum. Horseradish peroxidase-conjugated cortisol (200 µL) was added to each well on the ELISA plate and incubated on a mixer for 60 minutes. After emptying well contents, plates were washed 3 times with wash solution (400 µL/well) using an ELx50 plate washer (BioTek; Winooski, Vermont, USA). Residual wash solution was removed before 200 µL of tetramethylbenzidine (TMB) substrate solution was added to each well. The plate was then incubated on a mixer for 30 minutes. The reaction was stopped by adding 400 µL of 0.5M H₂SO₄ stop solution and then read at 450 nm using an Epoch microplate spectrophotometer (BioTek; Winooski, Vermont, USA) and calculated using Gen5 software (BioTek; Winooski, Vermont, USA). Cortisol concentrations (ng/mL) were calculated from the optical densities by the Gen5 software using 4-parameter logistic regression.

Fifty-two of the 60 children with usable fMRI data also provided usable cortisol data from 5 or more samples during the stress reactivity task. Of the 8 children without usable cortisol data, 5 were unable to produce saliva sufficient for measurement and 3 took 30 minutes or longer to produce their baseline cortisol sample. Of the 52 children with cortisol data, 1 child provided 5 samples, 6 children provided 6 samples, and 45 children provided 7 samples. Children with missing samples were required to have one of the first two samples (prior to frustration task and immediately after the frustration task [2 were missing the first sample and 4 were missing the second sample]) and the last sample. Number of saliva samples was not related to AUCₖ (r = .01, p = .9) and the mediation models reported in the main manuscript remained significant when number of cortisol samples was included as a covariate (PROCESS Indirect Effect [10,000 bootstrap samples]: .19 (.11), bias corrected 95% CI: .04/.5) and when children with fewer than 7
cortisol samples are excluded (PROCESS Indirect Effect [10,000 bootstrap samples]: .17 (.12), bias corrected 95% CI: .03/.5).

**Stress Evoked Cortisol Response in Young Children**

While cortisol scores increased at the group level between cortisol measures 1 (baseline mean = .5 [.07]) and 6 (mean peak at 40 minutes post stressor = .52 [.1]) following our stressor paradigm, this increase did not reach statistical significance (t = -.88, p = .39). Previous research examining stress evoked cortisol response in preschool age children has reported mean level cortisol increases, decreases, as well as significant variability at the individual level (e.g., some children exhibiting increases in cortisol and others exhibiting decreases in cortisol despite mean level increases or decreases at the group level). As an illustrative example, Tolep & Dougherty (8) recently reported an overall mean decrease in preschooler cortisol reactivity following exposure to the same in-lab stressor paradigm used in the current study. However, when cortisol increases were examined at the individual child level (i.e., peak value that was used to calculate increase was child specific), the authors found a stress-invoked cortisol response in 51.3% of their preschoolers. Similarly, despite reporting a mean level increase at the group level, De Weerth et al. (9) found that a significant minority of their preschoolers (39%) did not exhibit an increase in cortisol following an age appropriate, in-lab stressor paradigm. In line with the current study’s focus on individual differences, this research has also suggested that variability stress-evoked cortisol response following a stressor may be related to inter-individual differences in emotion regulation at this early age (e.g., high negative affect, low positive affect, etc) (8, 10, 11). Importantly, similar research in adults has suggested that individual variability in stress evoked cortisol response may be meaningfully related to brain activity. More specifically, attenuated
amygdala reactivity has been associated with a heightened stress evoked cortisol response following a psychosocial stressor in adults (12). Given the previous research reviewed above, it’s likely that variability in stress evoked cortisol response in preschoolers also reflects meaningful individual differences in stress reactivity and response that can be uniquely informative when interpreted within a broader context (i.e., not group mean(s) alone), including measures of brain function and emotion like those used in the current study.

Specificity of Findings to AUC$_g$

The current study included a measure of the overall stress evoked cortisol response (i.e., the amount of cortisol produced following the in-lab stressor) referred to as Area Under the Curve with respect to ground (AUC$_g$). This choice was based on previous research indicating that total cortisol output, rather than reactivity, following a stressor is associated with depression and depression risk (13, 14). Nevertheless, to provide additional support for the specificity of the reported findings to AUC$_g$, we re-ran our primary mediation analysis using AUC$_i$, a measure of cortisol change from baseline following a stressor (15). When using AUC$_i$, the mediation model including AUC$_i$ as the independent variable, amygdala reactivity as the mediator, and depression severity as the outcome was not significant (PROCESS Indirect Effect [10,000 bootstrap samples]: -.06 (.21), bias corrected 95% CI: -.51/.31).

Specificity of Findings to Depression Severity

To further bolster the specificity of our mediation findings to depression severity, we replaced PFC-S scores with raw scores from the CBCL (7) DSM-Oriented Anxiety and ODD subscales in our mediation model. CBCL scores were available for 50 of the 52 preschoolers included in the
current study. Mediation models including CBCL Anxiety and ODD raw scores as the outcome measure (rather than PFC-S scores) did not reach significance. In addition, providing additional support for the specificity of our findings to depression severity, amygdala reactivity continued to mediate the relationship between cortisol response (AUCg) and PFC-S scores when CBCL Anxiety and ODD subscale raw scores were included as covariates in the model (PROCESS Indirect Effect [10,000 bootstrap samples]: .18 (.11), bias corrected 95% CI: .04/.49). The CBCL Affective Problems subscale primarily includes items measuring symptoms of depression, such as sadness, irritability, diminished positive affect, sleep disturbances, altered eating, and fatigue. Given the strong positive correlation (r = .727, p < .001) between PFC-S scores and CBCL Affective Problems subscale raw scores, we replaced PFC-S scores with CBCL Affective Problems subscale raw scores in our mediation model to investigate whether our findings would replicate with this closely aligned measure. The mediation model including CBCL Affective Problems in place of PFC-S scores was significant (PROCESS Indirect Effect [10,000 bootstrap samples]: .05 (.03), bias corrected 95% CI: .0023/.1327), though with a reduced effect size. Collectively, these findings further support the specificity of our findings to depressive symptoms.

Results of Mediation Model When Excluding Two Children Without Reaction Time Data, When Not Controlling for Maternal Depression, and When Using Right Amygdala Reactivity to Outcome

In order to further establish the robustness of the mediation model supporting diminished amygdala reactivity to reward outcome as a mediator of the relationship between stress evoked cortisol response and depression, we reran our mediation analyses excluding the two children who did not make a button press during the CGT and when not controlling for maternal depression. The
reported results stay the same when maternal depression scores are not included as a covariate, including a significant positive relationship between AUCg and PFC-S scores ($r = .244$, $p = .04$), significant negative relationship between left amygdala reactivity and PFC-S scores ($r = -.36$, $p = .004$), and a significant mediation effect of amygdala reactivity (PROCESS Indirect Effect [10,000 bootstrap samples]: .24 (.15), bias corrected 95% CI: .04/.6).

Our mediation analyses also remained significant when the two children who did not make a button press during the CGT were excluded, (PROCESS Indirect Effect [10,000 bootstrap samples]: .21 (.12), bias corrected 95% CI: .04/.55).

Our primary analyses supported left amygdala reactivity to highly salient reward outcomes as an important mediator of the relationship between stress evoked cortisol response and depression severity in preschoolers. To further examine the specificity of our results to amygdala reactivity to highly salient reward outcomes versus amygdala responsivity in general, we carried out an additional mediation model using reactivity scores from the right amygdala region of interest, which exhibited a main effect of time (i.e., similar reactivity to all 3 outcomes) but not an outcome-by-time interaction. The mediation model using the average reactivity score across all outcomes relative to baseline (i.e., the percent signal change score representing a main effect of time) from the right amygdala as the mediator was not significant (PROCESS Indirect Effect [10,000 bootstrap samples]: -.0038 (.06), bias corrected 95% CI: -.14/.1), providing additional support for amygdala reactivity to highly salient outcomes as an important mediator of stress and depression in preschool age children.”

**Alternative Mediation Model**

A growing body of research suggests that early alterations in the developing stress system affect
neural response to reward and increase risk for depression as a result (16). We investigated a mediational model based on this research including stress evoked cortisol response as an independent variable (measured via AUC$_g$), amygdala reactivity to reward as a mediator variable, depression severity as an outcome variable, and relevant covariates (maternal depression, age in months, gender, CBCL anxiety raw score, CBCL, ODD raw score; please see parent paper and Specificity of Findings to Depression Severity subsection in the supplemental material). As predicted and in line with previous developmental theories and data, the tested mediation model was significant in our preschool aged sample, indicating that attenuated amygdala response to reward mediated the relationship between heightened stress evoked cortisol response and increased depression severity at this very early age (please see results in parent paper). However, given that our measures of stress evoked cortisol response, depression severity, and brain function were measured concurrently, the current data cannot inform directions of causality. As a result, it is possible that alternative mediational relationships between the included variables may be present and fit the current data. In line with this, we tested an additional mediation model based on the growing body of research suggesting a complex and reciprocal relationship between stress and depression (i.e., stress generation (17)). Following this line of thinking we tested two alternative mediation models including one placing stress evoked cortisol response as the independent variable, child depression severity as the mediating variable, and amygdala reactivity to reward as the outcome variable (model 1) and another placing depression as the independent variable, stress evoked cortisol response as the mediator variable, and amygdala reactivity to reward as the outcome variable (model 2). Both mediation models were not significant (Model 1: PROCESS Indirect Effect [10,000 bootstrap samples]: -0.004 (.004), bias corrected 95% CI: -0.02/.0007; Model 2: PROCESS Indirect Effect [10,000 bootstrap samples]: -0.003 (.003), bias corrected 95%
CI: -0.01/.0009).

**fMRI Data Acquisition Parameters and Preprocessing Methods**

Image acquisition included an initial low-resolution 3D sagittal T1-weighted MP-RAGE rapidly warped to Talairach space (18). This image was then used to provide on-line slice localization for the functional images, placing them as close as possible to the target template. T1 images (TR = 2,400 ms, TE = 3.16 ms, flip angle = 8°, slab = 176 mm, 176 slices, matrix size = 256 x 256, field of view (FOV) = 256 mm, voxel size = 1 x 1 x 1 mm, sagittal plan acquisition) were acquired as part of the structural imaging protocol and used in the transformation of images to a common template space optimized for preschool children (18). The accuracy and validity of this transformation for preschool age children has been demonstrated in previous research (19) and was confirmed through visual inspection for distortions and the accuracy of alignment for key cortical and subcortical landmarks. The functional images were collected with a 12-channel head coil using an asymmetric spin-echo echo-planar sequence sensitive to BOLD contrast (T2*) (TR=2000ms, TE=27ms, FOV=384mm, flip=90°). During each functional run, sets of 32 contiguous axial images with isotropic voxels (4mm³) were acquired parallel to the anterior-posterior commissure plane.

Prior to preprocessing, the first 4 frames of each run were discarded to allow for signal stabilization. The fMRI data were preprocessed and analyzed using in-house Washington University software. Data were reconstructed into images and normalized across runs by scaling whole-brain signal intensity to a fixed value and removing the linear slope on a voxel-by-voxel basis to counteract effects of drift (20). Data was also corrected for head motion using rigid-body rotation and translation correction algorithms (21-23), co-registered to Talairach space using a 12
parameter linear (affine) transformation that included resampling to 3mm cubic, and smoothed using a 6mm FWHM Gaussian filter. Within scan head movement was assessed using output from the rigid-body rotation and translation algorithm. After measuring the translations and rotations in the x, y, and z planes across frames, total root mean square (RMS) linear and angular measures were calculated and used to obtain the average amount of movement in millimeters per frame (i.e., 1 TR) in a given run for each subject (RMS/frame). CGT runs with greater than 1.5mm RMS/frame on average were excluded from further data analysis. Using this criterion, 64 children provided usable CGT data from only 1 of the 2 possible runs. To further reduce any potential effects of head movement on data quality, custom MATLAB (The Mathworks, Natwick, MA) code was used to identify frames with greater than 1mm absolute movement. The identified frames were removed from further data analysis (average percentage of frames removed = 11%). Four children with fewer than 65% of frames remaining after frame-by-frame censoring were not included in subsequent data analyses. Not surprisingly, children who did not provide usable MRI data (n=28; NMRI) were significantly younger on average (mean age 60 [11.5] months) than those who did (n=60; mean age 71 [9] months; MRI). However, groups did not differ in sex (NMRI 16 vs. MRI 33 females; \(x^2 = .04, p = .85\)), maternal BDI-II score (NMRI 7.3[7.8] vs. MRI 8.2[9.4]; \(t_{86} = .4, p = .69\)), or PFC-Scale score (NMRI 18.3[10.7] vs. MRI 15.8[10.5]; \(t_{86} = -1.1, p = .3\)).

Analyses using our a priori reward processing mask and at the whole brain were corrected for multiple comparisons using recommended guidelines (24) addressing recently identified challenges with inflated false positive rates in fMRI studies (25), the Analysis of Functional Neuro-Images (AFNI) 3dFWHMx and 3dClustSim commands were used to determine the combined p-value/cluster size thresholds required to maintain a false positive rate of \(p < .05\). Thresholds were \(z = 3\) (\(p < .001\)) and 10 voxels within our reward processing mask (correcting for all ROIs
simultaneously; false positive rate of \( p<.05 \) for the whole ROI mask) and \( z = 3 \) (\( p<.001 \)) and 26 voxels for whole-brain analyses (whole-brain false positive rate of \( p<.05 \)). Large clusters spanning multiple regions identified within our a priori reward processing mask were subsequently partitioned such that peaks of activity were considered separate regions if they were more than 12 mm apart, as measured by a peak-splitting algorithm. Individual time courses within the identified regions were then extracted for subsequent analyses using 6mm spheres centered at the peak voxel coordinates.

**fMRI Task**

Prior to the child gambling task (CGT), children are asked to choose which of two different candies (M&Ms or Skittles) they would like to play for during the CGT. Once they have chosen their candy, they are introduced to how the CGT is played during their in-person assessment using child-friendly language to describe how it is played (e.g., “Your job is to guess if the person hiding behind the question mark is bigger or smaller than you!”), by providing examples of the CGT images and testing understanding of them during instruction (“This is an adult. Are they bigger or smaller than you?”), and providing children a sequence of practice trials (fixation cross, question mark, outcome …) where they play the CGT and demonstrate their understanding of it (on average ~5-10 trials). Each child practices until they can demonstrate they know when to guess and how to make a guess (e.g., press the button for bigger don’t press it for smaller). In addition, prior to playing the CGT in the scanner each child is reminded of how the game is played and asked to demonstrate a verbal understanding of how to respond (e.g., Staff: “What do you do when you want to guess bigger?”, Child: “Press the button.”).
fMRI Whole Brain Results

Whole brain results were significant for a main effect of time in multiple cortical and subcortical regions (see Figure S1 and Table S1). Follow-up analyses found outcome x time effects in parahippocampal gyrus, fusiform gyrus and postcentral gyrus (see Table S1). A large cluster within the right fusiform gyrus was subsequently partitioned such that peaks of activity were considered separate regions if they were more than 12 mm apart, as measured by a peak-splitting algorithm. Additional cortical and subcortical regions were identified following this process (see Table S1). The region matching closest to our a priori region of interest in the left amygdala spanned portions of the amygdala as well as the uncus. Similar to the results reported for the amygdala in the main text, a negative correlation between reactivity within the larger amygdala/uncus ROI (using gain/loss minus neutral difference scores created in a fashion identical to our primary amygdala ROI analyses) and depression severity was found ($r = -.38$, $p = .007$). However, while in the expected direction, the correlation between amygdala/uncus reactivity and stress evoked cortisol response ($AUC_g$) did not reach significance ($r = -.13$, $p = .17$), suggesting specificity of this relationship to the amygdala.
Table S1: Whole Brain Analyses: Regions identified with main effect of time and outcome x time interaction

<table>
<thead>
<tr>
<th>Region</th>
<th>Hemisphere</th>
<th>BA</th>
<th>Peak Voxel</th>
<th>Cluster (voxels)</th>
<th>Outcome x Time</th>
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*Peak splitting results using a required cluster distance of 30mm are reported underneath the original cluster.
Figure S1: Regions identified with a significant main effect of time during the child gambling task. Colored bar at right represents z-value at the individual voxel level.
Supplemental References


