SUPPORTING APPENDIX

Early Developmental Emergence of Human Amygdala-Prefrontal Connectivity after Maternal Deprivation

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Supporting Methods

Demographic Characteristics

Participants consisted of 89 children and adolescents (41 PI children and 48 comparison, never-institutionalized children; Table S1). All participants were right-handed. All comparison participants were physically and psychiatrically healthy (no medical or psychiatric disorders), as confirmed by a telephone screening prior to participation. Pubertal status was measured using the Pubertal Scale of Development (parent-report) for 40 comparison participants and 21 PI participants (1). Cognitive ability was assessed using the Wechsler Abbreviated Scale of Intelligence (2), with the average full-scale intelligence quotient of the sample within the average range (mean=106.5; S.D.=17.2). Both groups were characterized by a modal caregiver education level of a 4-year college degree and an annual income above the median annual household income in the United States (percentage of caregivers with income > $100,000 per year: 45% comparison, 62% PI). PI children were from Asian American (52%) and European American (48%) backgrounds. Comparison children were from European American (34%), African American (25%), multiracial (23%), Asian American (11%), and Latino (8%) backgrounds.

Table S1. Characteristics of previously institutionalized (PI) and comparison participants

	PI (n=41)	**Comparison (n=48)**	
Sex	27 female (64%); 15 male (36%)	23 female (57%); 31 male (43%)	$X^2(1)=3.628$, *ns* (p=.06)
Mean age in years (SD); range	10.8 (2.7); 6.5-16.6	12.1 (3.3); 6.5-17.6	t(94)=2.01, p=.048
Mean (SD) IQ; range	102.5 (17.2); 69-148	112.0 (18.1); 76-148	t(94)=2.06, p=.042
Pubertal Status	2.4 (0.9); 1-4	2.3 (0.9); 1-4	t(59)=0.31, p=.76
Country of origin	2 Azerbaijan, 14 China, 1 India, 7 Kazakhstan, 11 Russia, 1 Slovak Republic, 4 Ukraine (2 unknown)		
Mean age orphaned in months (SD); range	6.2 (9.6); 0-36		
Mean age adopted in months (SD); range	28.1 (23.4); 2-96		
Mean time in orphanage in months (SD); range	21.8 (17.5); 2-72		
Mean time with family in months (SD); range	95.5 (38.6); 31-185		

Amygdala Functional Connectivity

For the PPI analysis, a GLM analysis was carried out in AFNI for each participant with four regressors for task, one for seed region timeseries, four for the interaction of task and timeseries, one for accuracy, and six motion regressors. The four psychological (task) regressors modeled whether a given trial consisted of viewing an emotional face (i.e., fearful, happy, neutral in fear run, and neutral in the happy run) or fixation. The physiological (seed region timeseries) regressor comprised the timeseries for the right amygdala, as defined anatomically in Talairach space. The four interaction regressors modeled the interaction of the psychological regressors and the physiological regressor, such that each interaction regressor identified regions whose timeseries correlated in a task-dependent manner with the amygdala timeseries. The GLM analyses fit the percent signal change time courses to each regressor, and linear and quadratic trends were modeled for each voxel's time course to control for correlated drift. One outlier participant was excluded due to amygdala connectivity three standard deviations beyond the mean. All findings remained the same when analyses were conducted with and without the outlier, thus results are reported on the analyses excluding the outlier.

The individual-level regression coefficients were then submitted to random-effects, group level analyses. Consistent with the analysis of amygdala reactivity, an ANOVA analysis was conducted in AFNI to model group (i.e., comparison, PI), emotional run (i.e., fear run, happy run), and stimulus type (i.e., emotional face, neutral face). We tested for main effects and interactions between group, emotional run, and stimulus type. Correction for multiple comparisons was applied at the cluster level following Monte Carlo simulations conducted in the AlphaSim program within AFNI.

Motion

Systematic procedures were implemented to reduce motion, particularly in younger participants, and to ensure that children remained still throughout the duration of the task. Before the MRI scanning session, children participated in a mock scanning session to help them to acclimate to the scanning environment and to feel comfortable with the scanning procedures. In addition, this step provided an opportunity for children to practice and receive feedback on lying still in order to optimize children's ability to remain still during actual data collection. During data collection, an air vacuum pillow (Siemens Comfort Pack) was used to pad and secure the child's head in a comfortable, steady position. Additional padding was placed around the child's head. In addition, all participants were provided with feedback and reminders regarding motion throughout the scanning session.

Multiple steps were taken to correct for motion. All analyzed data were free of motion greater than 2.5mm in any direction. Volumes with motion greater than 2.5mm in any direction were excluded (via censoring), and all participants had fewer than 30% of total volumes censored (mean % of censored volumes=3.0%; mode=0%). Preprocessing included standard spatial realignment to correct for motion. Motion regressors were included in our imaging analyses (at the subject level, motion in all six directions at the trial by trial level). In addition, multiple analyses were conducted to rule out potential effects of motion. For each participant, we calculated the mean displacement value (3) and average motion across six directions. Specifically, we tested whether mean displacement value or average motion differed between the comparison and PI groups and whether these measures of motion related to age, amygdala activation, or amygdala-mPFC functional connectivity.

Given recent advances in methods for controlling for motion, we also conducted a secondary analysis in which we re-analyzed our functional connectivity data controlling for different motion levels across participants (3). In AFNI, we performed the original whole-brain group-level regression of amygdala functional connectivity with mean displacement value as a covariate. In addition, we performed the regression of group, age, and a group by age interaction on amygdala-mPFC functional connectivity while controlling for mean displacement value. Finally, we performed a whole-brain regression of mean displacement value on amygdala functional connectivity to test for any relationships between motion and functional connectivity.

Cortisol Analyses

Participants provided saliva samples prior to and following the MRI scan. The time of collection prior to the MRI scan ranged from 8:45am to 5:30pm, with the average time of collection as 12:53pm (S.D. = 2 hours, 23 min); the time of collection following the MRI scan ranged from 10:00am to 7:05pm, with the average time of collection as 2:16pm (S.D. = 2 hours, 23 min). Salivette swabs (Sarstedt) were used to collect saliva samples. Samples were frozen immediately and stored at -20 degrees Celsius. Samples were packaged over dry ice and shipped using priority mail to Germany for processing. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary cortisol concentrations were measured using commercially available chemiluminescence-immuno-assays with high sensitivity (IBL International, Hamburg, Germany). The intra and interassay coefficients for cortisol were below 8%. Results were sent electronically. A mediational model was used to test whether salivary cortisol during recovery from a challenge (MRI scan) mediated the hypothesized relationship between maternal deprivation and amygdala-mPFC functional connectivity. Specifically, regression analyses were conducted to test the relationships between maternal deprivation, post-MRI salivary cortisol level, and functional connectivity. While evidence suggests that time of day does not affect changes in cortisol reactivity from baseline to psychosocial stress (4), all analyses controlled for the time of day at which the post-MRI salivary cortisol sample was obtained. To test for mediation while controlling for age given anticipated age-related differences in connectivity, standardized residuals for amygdala-mPFC connectivity were employed when age was regressed on connectivity.

A subsample (41 comparison, 24 PI) of participants also completed measures of cortisol at home. Participants collected four salivary samples (at wake-up, 45 minutes after wake-up, 5 p.m., and 8 p.m.) on two separate days. Participants were instructed to collect the samples on normal days when they were feeling healthy. In order to provide a reference point for the MRI-based cortisol samples, hierarchical linear modeling was used to estimate the expected value for cortisol for each participant at the time of pre-MRI cortisol collection and post-MRI cortisol collection. These statistical analyses were conducted using SAS 9.3 using PROC MIXED and full maximum likelihood estimation.

Behavioral Data Analyses

For each participant, we calculated the mean reaction time (RT) for correct hits to neutral faces for each run. D-prime, a measure of accuracy that accounts for response bias, was calculated by subtracting the z-transformed false alarm rate from the z-transformed hit rate. To examine effects of group, age, and brain function on behavioral performance, univariate GLMs were conducted in SPSS with factors for group, age amygdala reactivity (during the fear run), and amygdala-mPFC connectivity (during the fear run). The behavioral outcomes of interest were mean RT (for correct hits to neutral faces in the fear run) and d-prime (for the fear run). Behavioral data for three participants (2 comparison, 1 PI) was excluded due to technical difficulties with the button box.

Supporting Results

Brain Activation

In addition to the right amygdala, a group by emotional run interaction was also observed in a cluster located in right superior temporal gyrus and right inferior frontal gyrus ($t=19.39$, cluster: 1213 voxels, $p<.0001$, corrected; peak voxel: 59, -16, 2) (Figure S1; Table S2). Specifically, PI participants exhibited elevated activation relative to comparison participants for the fear run ($t(92)=-2.102$, $p=.038$) but not the happy run ($t(92)=.669$, $p=.51$). There were no group- or age-related differences in mPFC activation, based on the functional cluster in mPFC defined in the PPI analysis (Figure S2).

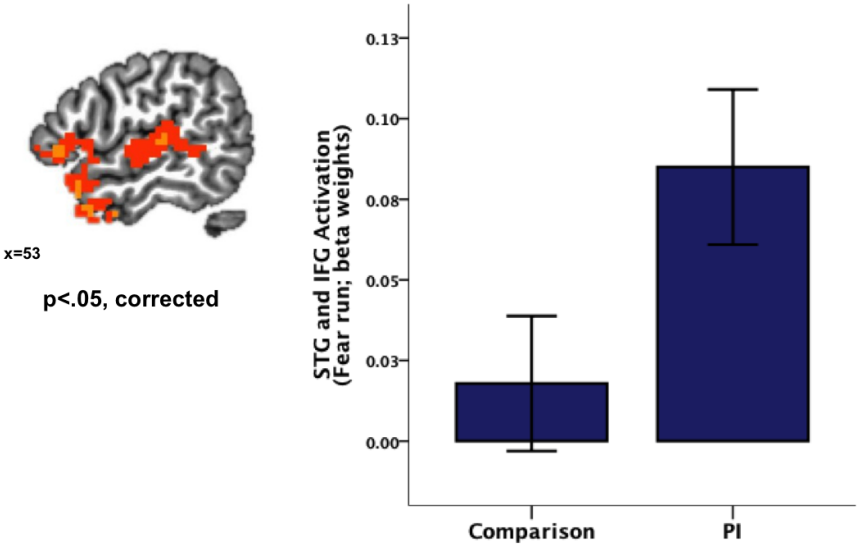

Figure S1. The PI group showed greater activation in the right superior temporal gyrus (STG) and inferior frontal gyrus (IFG) than the comparison group during the fear run. Errors bars: +/- 1 standard error of the mean.

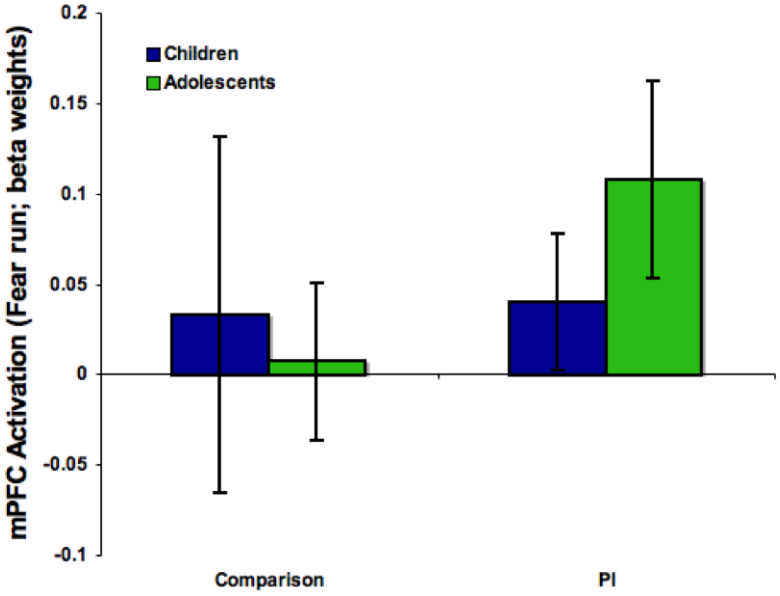

Figure S2. There were no group- or age-related differences in mPFC activation, based on the functional cluster in mPFC defined in the PPI analysis. Errors bars: +/- 1 standard error of the mean.

Table S2. Regions with group x emotion interaction for activation and functional connectivity

Region	Peak Voxel	Cluster Size	T value	P value (corrected)
Activation (Group x Emotional Run)				
Amygdala, parahippocampal gyrus, thalamus, caudate, lentiform nucleus (right)	(-1, 2, 2)	636	18.74	p<.0001
Superior temporal gyrus, inferior frontal gyrus (right)	(59, -16, 2)	1213	19.39	p<.0001
Functional Connectivity (Group x Emotional Run)				
Anterior cingulate (BA 32, 24, 10) (bilateral), middle frontal gyrus (bilateral), middle frontal gyrus (bilateral), medial frontal gyrus (left), superior frontal gyrus (left), inferior frontal gyrus (left)	(-3, 27, -1)	487	11.36	p<.0001

Amygdala-mPFC Functional Connectivity

In order to examine the effects of maternal deprivation on functional connectivity between the amygdala and prefrontal cortex, a PPI analysis was performed for each participant and analyzed at the group level using a whole-brain ANOVA in AFNI. A significant group x emotional run interaction was observed in bilateral prefrontal cortex (t=11.36, cluster: 487 voxels, p<.0001, corrected; peak voxel: -3, 27, -1), including anterior cingulate (BA 32, BA 24, BA 10). While PI participants showed stronger negative functional connectivity than comparison participants as a group for the fear run, a different pattern was found for the happy run. Specifically, comparison participants showed stronger negative functional connectivity relative to the PI group (Figure S3). Neither group exhibited an age-related change in connectivity to the happy run.

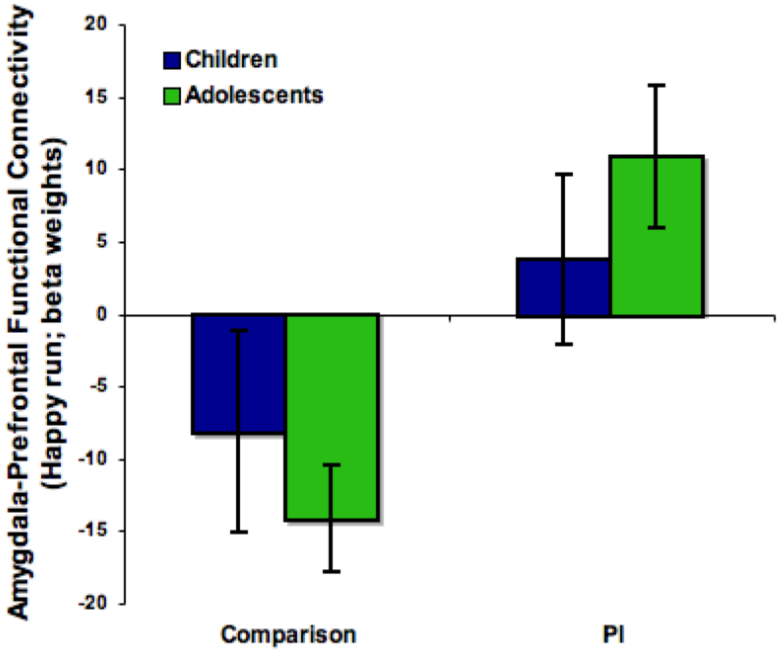

Figure S3. Whereas a shift from positive to negative functional connectivity was observed between comparison children and adolescents for the fear run, no age-related changes were observed to the happy run for either group. Errors bars: +/- 1 standard error of the mean.

Replication of Previous Results in Healthy Controls

Because some of the participants in the present sample also contributed to the prior study of typical development (Gee et al., 2013), we reanalyzed the present data excluding the 28 comparison participants who also contributed to the normative change reported in the prior study. The normative change (shift from positive to negative amygdala-mPFC functional connectivity) replicated in the non-overlapping sample (Figure S4). Similar to the original results reported in the present study, there was a significant group x age interaction in functional connectivity (F(6,66)=5.35, p=.03). Specifically, connectivity shifted from positive to negative between comparison children and adolescents, whereas connectivity was negative in both PI children and adolescents. Within the comparison group, the difference in connectivity between comparison children and adolescents was still significant (t(23)=2.38, p=.026) in the non-overlapping sample.

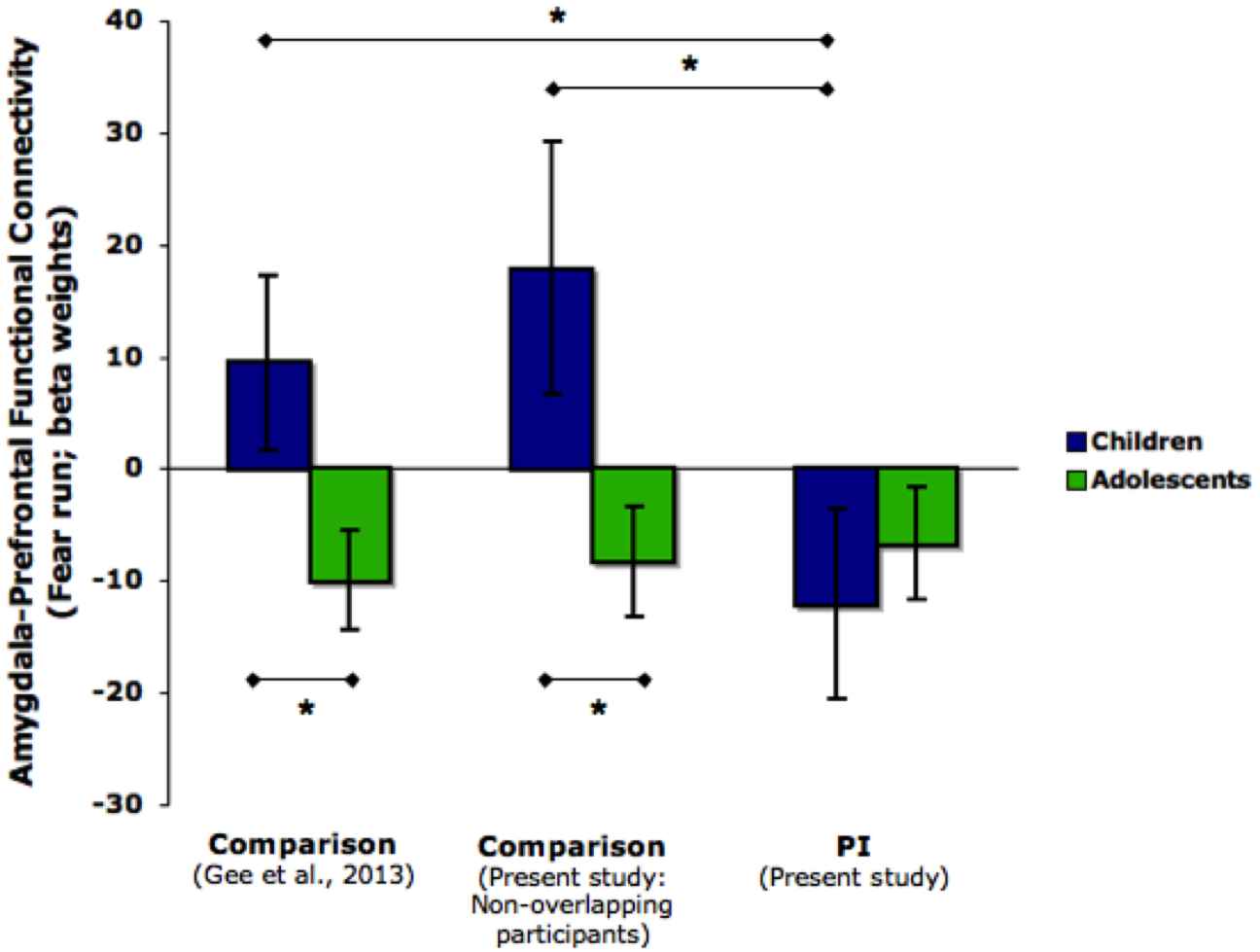

Figure S4. In previous work, we observed a shift from positive to negative amygdala-prefrontal functional connectivity in typical development (Gee et al., 2013). Due to the overlap between some comparison participants in that study and the present investigation, we also tested for a replication of the normative change in connectivity, and of the group by age interaction with the non-overlapping comparison participants. Results replicated, such that there was again a significant difference in functional connectivity between comparison children and adolescents in the current study (with non-overlapping participants). In addition, we show a replication of the group by age interaction using the non-overlapping sample of comparison participants, such that the comparison group showed the expected developmental switch but the PI group demonstrated negative connectivity by childhood. Errors bars: +/- 1 standard error of the mean.

Motion

Mean displacement value (Van Dijk et al., 2012) and average motion did not differ between groups ($p=.96$, $p=.27$, respectively). Moreover, they did not relate to age, amygdala reactivity, or amygdala-mPFC functional connectivity (all $ps>.05$). When we conducted the original whole-brain regression of amygdala functional connectivity covarying for mean displacement value, the results replicated our original findings of connectivity in mPFC ($t=11.16$, $p<.0001$). We further performed the regression of group, age, and a group by age interaction on amygdala-mPFC functional connectivity while controlling for mean displacement value. Mean displacement value was not significant in the model ($p=.99$), and the group by age interaction for functional connectivity remained significant ($F(13,94)=3.63$, $p=.011$). Lastly, the whole-brain regression of mean displacement value on amygdala functional connectivity showed only two clusters, which were located in right middle temporal gyrus and cerebellum.

Controlling for IQ

Though comparison (mean=103.97, S.D.=13.62) and PI (mean=104.48, S.D.=19.32) adolescents did not differ on IQ ($t(55)=0.89$, $p=.378$), comparison (mean=121.11, S.D.=16.88) and PI (mean=104.48, S.D.=19.32) children differed on IQ ($t(37)=2.84$, $p=.007$). Thus, we reanalyzed our data controlling for IQ. All primary findings held when IQ was included as a covariate. Specifically, there was a group by age interaction for amygdala-mPFC functional connectivity that remained significant ($p=.020$) over and above the effect of IQ ($p=.580$). The group difference in amygdala reactivity also remained significant ($p=.020$) over and above the effect of IQ ($p=.398$). The group by connectivity valence interaction for separation anxiety remained significant ($p=.035$) over and above the effect of IQ ($p=.001$). Finally, the group difference in post-MRI cortisol remained at trend level ($p=.079$) over and above the effect of IQ ($p=.479$).

Cortisol Measures

For the subsample of participants who also provided salivary samples collected at home, we were able to test for potential differences between estimated cortisol values at home and actual cortisol values in the scanning environment. Pre- and post-MRI cortisol did not differ from estimated at-home values for comparison (pre-MRI: $F(1,39)=0.54$, $p=.467$; post-MRI: $F(1,39)=0.10$, $p=.920$) or PI participants (pre-MRI: $F(1,22)=0.54$, $p=.818$; post-MRI: $F(1,22)=2.42$, $p=.134$). In addition, we used repeated measures ANOVA (controlling for age) to test for differences between actual pre- and post-MRI cortisol. Although not significant, there was a trend toward a decrease in cortisol from pre- to post-MRI in controls (pre-MRI: mean=8.19, S.D.=8.32; post-MRI: mean=7.34, S.D.=5.72), but cortisol did not differ pre- and post-MRI in the PI participants (pre-MRI: mean=8.48, S.D.=8.40; post-MRI: mean=10.07, S.D.=7.01).

Behavioral Performance

To examine effects of group, age, amygdala reactivity, and functional connectivity on behavioral performance, univariate GLMs were conducted in SPSS with factors for group, age, amygdala reactivity, and amygdala-mPFC connectivity (during the fear run). For RT, a main effect of age was observed such that participants responded more quickly with increased age ($F(1,91)=7.94$, $p=.006$). In addition, a main effect of connectivity was observed, such that reaction time became faster as amygdala-mPFC connectivity became more strongly negative ($F(1,91)=4.77$, $p=.032$). Within groups, the relationship between faster RT and increased age held for the comparison group ($r=.41$, $p=.003$), but not for the PI group ($r=.08$, $p=.635$). For d-prime, a main effect of age was also observed, such that increased age was associated with higher d-prime values ($F(1,91)=14.24$, $p<.0001$). For descriptive purposes, participants were divided into younger and older groups, with performance for each age group presented for d-prime and RT during the fear run (Table S3). These performance measures indicated that comparison and PI participants of all ages were able to understand and attend to the task, supporting its use with this developmental population.

Table S3. Behavioral performance by age group

	Comparison Children (n=17)	**Comparison Adolescents** (n=35)	**PI Children** (n=19)	**PI Adolescents** (n=21)
d-prime (for fear run)	1.92 (1.37)	2.89 (1.64)	2.33 (1.51)	3.33 (1.76)
RT (Hits to neutral faces in fear run; msec)	718 (197)	627 (171)	768 (187)	616 (118)

Medication Status

A subset of PI participants had current medication use (Table S4).

Table S4. Medication use in PI participants

Medication Status	**PI Participants (n)**
No current medication	29 (69%)
One current medication	9 (21%)
Two current medications	2 (5%)
Three current medications	2 (5%)
Psychostimulant	9 (21%)
Antidepressant	2 (5%)
Other (e.g., Abilify, BuSpar, Seroquel)	3 (7%)

In order to examine whether medication use influenced our results, secondary analyses were performed to covary for medication status in all of our original analyses. Medication status was not significant in the model of amygdala reactivity (p=.62), and our original finding of amygdala hyperreactivity in PI participants remained significant ($F(1,94)=5.12$, p=.026). Similarly, medication status was not significant in the model of amygdala-mPFC functional connectivity (p=.81), and the original finding of a group x age interaction in functional connectivity remained significant ($F(13,94)=3.35$, p=.016). Main effects of group ($F(1,90)=29.11$, $p<.0001$) and a group x connectivity valence interaction ($F(1,90)=4.29$, p=.041) on separation anxiety remained, and there was no effect of medication status on separation anxiety (p=.92). In addition, when medication status was included in the GLM model of separation anxiety, a main effect of valence shifted toward trend level significance ($F(1,90)=2.95$, p=.09). For the mediation analyses, medication status was not associated with post-MRI cortisol (p=.45), and the relationship between maternal deprivation and post-MRI cortisol was at trend level (B=.177, p=.143) when medication status was included in the regression. Similarly, medication status was not associated with connectivity (p=.38), and the relationship between post-MRI cortisol and connectivity remained significant (B=-.265, p=.017) when medication status was included in the regression. The relationship between maternal deprivation and connectivity remained at trend level (p=.120) when medication status was in the regression and showed a substantial decrease in strength when post-MRI cortisol was included in the model (p=.358), similar to the original results of the mediation analysis. Medication status was also not significant in the model of RT, and main effects of age ($F(1,91)=7.56$, p=.007) and functional connectivity ($F(1,91)=4.38$, p=.039) on RT remained significant. In the GLM for d-prime, the main effect of age ($F(1,91)=15.66$, $p<.0001$) remained significant when controlling for medication status in the model. Medication status was significantly related to d-prime in the model ($F(1,91)=4.36$, p=.04), and group became significant ($F(1,91)=5.01$, p=.028), such that d-prime values were higher for the PI than comparison group.

In addition to covarying for medication status in our models, we specifically tested whether medication status was associated with any variables of interest to further rule out effects of medication on our findings. Independent sample t-tests comparing PI participants with (n=28) versus without (n=13) medication use demonstrated that medication status did not relate to amygdala reactivity, activation in the cluster located in superior temporal gyrus and inferior frontal gyrus, amygdala-mPFC functional connectivity, age, separation anxiety, salivary cortisol level post-MRI scan, RT, or d-prime (all *p*s>.05).

Finally, secondary analyses were conducted excluding all participants taking medications. Of note, all findings related to amygdala-prefrontal circuitry remained despite a loss of statistical power when excluding medicated participants. Specifically, results replicated our original finding of a group x age interaction in amygdala-mPFC functional connectivity (p=.009; Figure S5). In addition, results replicated our original findings of elevated reactivity among PI participants in the amygdala, superior temporal gyrus, and inferior frontal gyrus (p=.033; Figure S6). In the GLM for separation anxiety, the main effect

of group remained significant; however, the group x valence interaction was not significant (p=.26), perhaps due to a loss of power (Figure S7). When medicated participants were excluded, the relationships between maternal deprivation and post-MRI cortisol (p=.239), and between post-MRI cortisol and connectivity (p=.149), did not remain significant (Figure S8). The relationship between maternal deprivation and connectivity was at trend level (p=.126) and showed a substantial decrease in strength when post-MRI cortisol was in the model (p=.318), similar to the original results of the mediation analysis. In behavioral analyses, main effects of age replicated for RT and d-prime. In the GLM for d-prime, group became significant when participants on medication were excluded, such that d-prime was higher for PI than comparison participants.

Age of adoption did not relate to amygdala reactivity, amygdala-mPFC functional connectivity, cortisol level, or separation anxiety (all *p*s>.05).

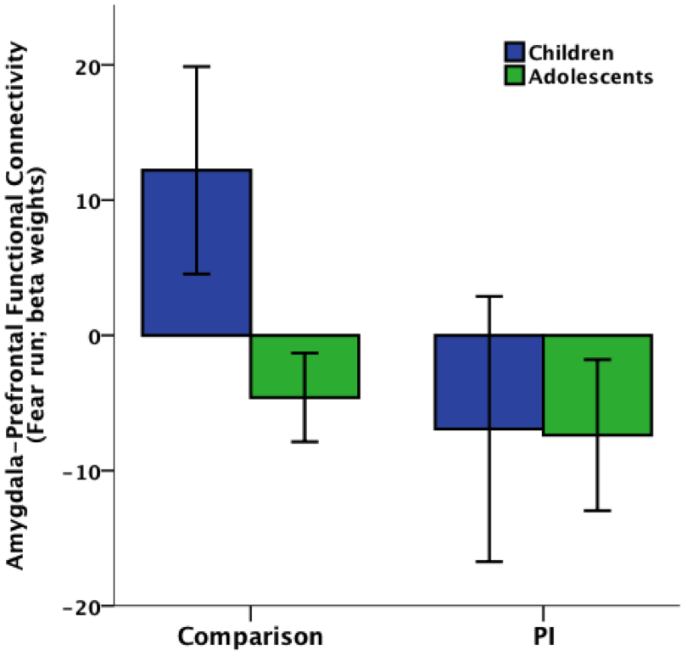

Figure S5. When medicated participants were excluded, results replicated the group by age interaction for amygdala-mPFC functional connectivity. Specifically, the comparison, but not PI, group showed a developmental switch from positive to negative coupling. Errors bars: +/- 1 standard error of the mean.

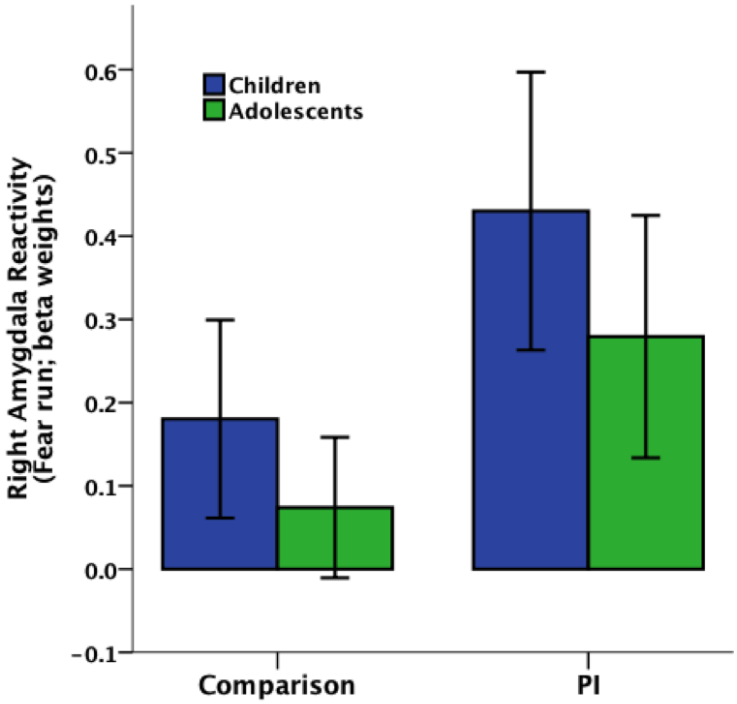

Figure S6. When medicated participants were excluded, the group difference in amygdala reactivity replicated, such that the PI group exhibited greater amygdala reactivity than the comparison group. Errors bars: +/- 1 standard error of the mean.

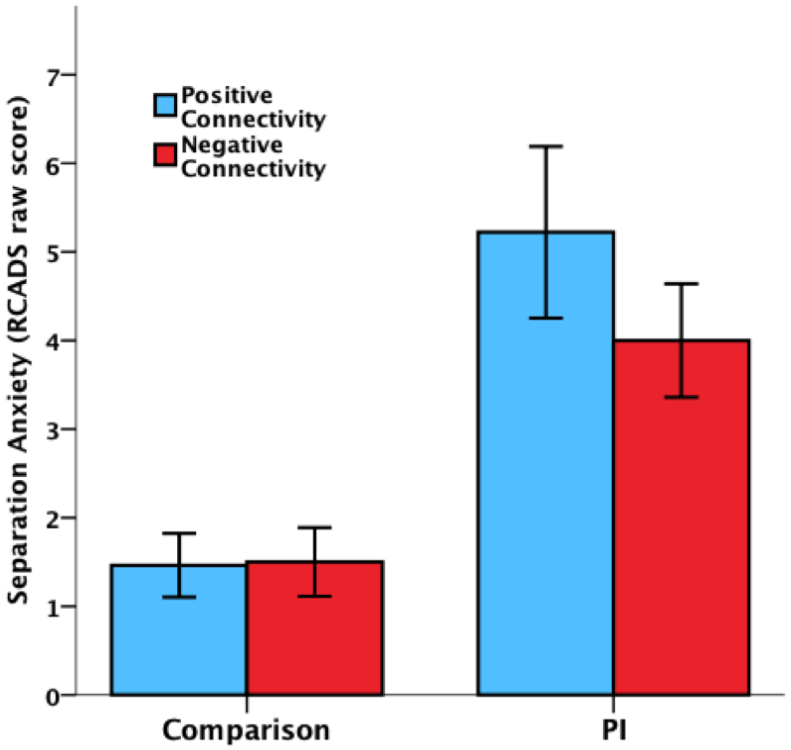

Figure S7. Separation anxiety and connectivity valence, excluding medicated participants. The main effect of group remained significant. Errors bars: +/- 1 standard error of the mean.

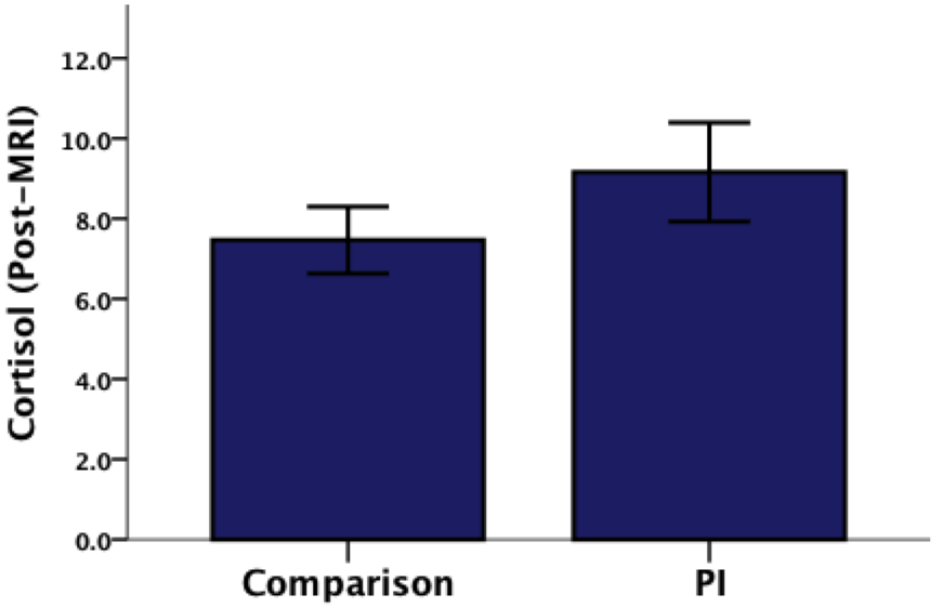

Figure S8. Post-MRI cortisol by group, excluding medicated participants. The direction of effect remained; however, the group differences was not significant, perhaps due to a loss of power. Errors bars: +/- 1 standard error of the mean.

Supporting References

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