

ORIGINAL ARTICLE

# Evolutionarily conserved prefrontal-amygdalar dysfunction in early-life anxiety

RM Birn<sup>1,2,3,4,5,12</sup>, AJ Shackman<sup>6,7,8,12</sup>, JA Oler<sup>2,3,4</sup>, LE Williams<sup>2,3,4</sup>, DR McFarlin<sup>2,3,4,5</sup>, GM Rogers<sup>2</sup>, SE Shelton<sup>2</sup>, AL Alexander<sup>1,5</sup>, DS Pine<sup>9</sup>, MJ Slattery<sup>2</sup>, RJ Davidson<sup>2,3,5,10,11</sup>, AS Fox<sup>2,3,4,5,10,11</sup> and NH Kalin<sup>2,3,4,5,10</sup>

Some individuals are endowed with a biology that renders them more reactive to novelty and potential threat. When extreme, this anxious temperament (AT) confers elevated risk for the development of anxiety, depression and substance abuse. These disorders are highly prevalent, debilitating and can be challenging to treat. The high-risk AT phenotype is expressed similarly in children and young monkeys and mechanistic work demonstrates that the central (Ce) nucleus of the amygdala is an important substrate. Although it is widely believed that the flow of information across the structural network connecting the Ce nucleus to other brain regions underlies primates' capacity for flexibly regulating anxiety, the functional architecture of this network has remained poorly understood. Here we used functional magnetic resonance imaging (fMRI) in anesthetized young monkeys and quietly resting children with anxiety disorders to identify an evolutionarily conserved pattern of functional connectivity relevant to early-life anxiety. Across primate species and levels of awareness, reduced functional connectivity between the dorsolateral prefrontal cortex, a region thought to play a central role in the control of cognition and emotion, and the Ce nucleus was associated with increased anxiety assessed outside the scanner. Importantly, high-resolution 18-fluorodeoxyglucose positron emission tomography imaging provided evidence that elevated Ce nucleus metabolism statistically mediates the association between prefrontal-amygdalar connectivity and elevated anxiety. These results provide new clues about the brain network underlying extreme early-life anxiety and set the stage for mechanistic work aimed at developing improved interventions for pediatric anxiety.

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## INTRODUCTION

Some children and animals characteristically show heightened reactions to novelty and potential threat.<sup>1,2</sup> When extreme, this anxious temperament (AT) is a well-established risk factor for the development of anxiety, depression and comorbid substance abuse.<sup>3,4</sup> These disorders are common, debilitating and difficult to treat.<sup>5–7</sup> As such, they represent a growing burden on public health-care systems and an important challenge for clinicians, health economists and public policy-makers.<sup>8,9</sup> AT is a trait-like phenotype that is determined by a combination of heritable and non-heritable factors, evident early in life and characterized by increased behavioral and hypothalamic–pituitary–adrenal reactivity to novelty and potential threat.<sup>3,10–13</sup> There is a considerable evidence that the high-risk AT phenotype is attenuated by anxiolytic compounds<sup>14,15</sup> and expressed similarly in children and young monkeys.<sup>3,10</sup> Importantly, mechanistic and neuroimaging studies in young monkeys demonstrate that metabolic activity in the central (Ce) nucleus of the amygdala is a key substrate for individual differences in early-life AT. In particular, selective excitotoxic lesions of the primate Ce nucleus reduce AT,<sup>16</sup> consistent with studies of human patients with circumscribed amygdala damage.<sup>17</sup> Furthermore, in young monkeys individual differences in Ce nucleus metabolism are

trait-like and predict substantial variation in the AT phenotype.<sup>10–13</sup> These observations are in accord with evidence that the Ce nucleus can initiate a broad spectrum of defensive responses via efferent projections to the brain regions that directly mediate many of the behavioral, physiological and cognitive features of anxiety.<sup>11,18,19</sup>

There is consensus that neuropsychiatric disorders, like other complex mental processes, reflect alterations in the coordinated activity of distributed functional circuits.<sup>20</sup> Anatomically, the Ce nucleus is embedded in a complex web of monosynaptically and polysynaptically connected brain regions.<sup>19</sup> This structural backbone encompasses a number of cortical regions that are especially well developed in primates, including the anterior cingulate cortex (ACC), insula and prefrontal cortex (PFC).<sup>21–23</sup> Although it widely believed that the synchronized flow of information across this network underlies primates' capacity for flexibly regulating anxiety, the functional architecture of the Ce nucleus network and its relevance to early-life anxiety remains poorly understood. This partially reflects the fact that functional networks need not recapitulate the direct structural connections revealed by traditional tract tracing techniques.<sup>24,25</sup> In particular, there is mounting evidence that regulatory signals can propagate across more complex, indirect pathways.<sup>26</sup>

<sup>1</sup>Department of Medical Physics, University of Wisconsin, Madison, WI, USA; <sup>2</sup>Department of Psychiatry, University of Wisconsin, Madison, WI, USA; <sup>3</sup>HealthEmotions Research Institute, Wisconsin Psychiatric Institute and Clinics, University of Wisconsin, Madison, WI, USA; <sup>4</sup>Lane Neuroimaging Laboratory, University of Wisconsin, Madison, WI, USA; <sup>5</sup>Waisman Laboratory for Brain Imaging and Behavior, University of Wisconsin, Madison, WI, USA; <sup>6</sup>Department of Psychology, University of Maryland, College Park, MD, USA; <sup>7</sup>Neuroscience and Cognitive Science Program, University of Maryland, College Park, MD, USA; <sup>8</sup>Maryland Neuroimaging Center, University of Maryland, College Park, MD, USA; <sup>9</sup>Section on Development and Affective Neuroscience, National Institute of Mental Health, Bethesda, MD, USA; <sup>10</sup>Department of Psychology, University of Wisconsin, Madison, WI, USA and <sup>11</sup>Center for Investigating Healthy Minds, University of Wisconsin, Madison, WI, USA. Correspondence: Dr NH Kalin, HealthEmotions Research Institute, Wisconsin Psychiatric Institute and Clinics, University of Wisconsin, 6001 Research Park Boulevard, Madison, 53719 WI, USA.  
E-mail: nkalin@wisc.edu

<sup>12</sup>These authors contributed equally to this work.

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Here, we used a combination of neuroimaging modalities to survey the entire brain for patterns of functional connectivity predictive of both the intermediate brain phenotype (Ce nucleus metabolism) and the high-risk phenotype (AT). Specifically, we used functional magnetic resonance imaging (fMRI) and high-resolution 18-fluorodeoxyglucose positron emission tomography (FDG-PET) to first trace the intrinsic functional connectivity of the Ce nucleus in anesthetized young monkeys and then to identify connectivity patterns associated with Ce nucleus metabolism and anxiety. fMRI-derived measures of intrinsic functional connectivity are particularly useful because they are sensitive to functional networks spanning polysynaptic circuits,<sup>27–29</sup> just as viral tracers can be used to delineate polysynaptic structural pathways.<sup>30</sup> Indeed, there is ample evidence of robust functional connectivity between brain regions that lack direct structural connections.<sup>28,29,31</sup> This is critical because the Ce nucleus is the major output region of the amygdala and receives relatively few direct projections from prefrontal regions implicated in the regulation of stress and anxiety.<sup>19,32–34</sup> Therefore, the regulation of Ce nucleus activity is likely to be mediated indirectly, via polysynaptic circuits spanning the PFC and neighboring regions of the amygdala with the capacity to convey cortical regulatory signals to the Ce nucleus.<sup>19,32–33</sup> Finally, to assess the relevance of our discoveries in the rhesus monkey to understanding childhood disease, we used fMRI to search for homologous patterns of intrinsic functional connectivity in children with anxiety disorders. Identifying an evolutionary conserved functional circuit relevant to extreme anxiety would reinforce the validity of the young rhesus model and set the stage for future mechanistic research aimed at understanding the origin of differences in the strength of functional connectivity.<sup>35,36</sup>

## MATERIALS AND METHODS

Except where noted otherwise, behavioral and brain imaging methods have been described in detail in prior publications and are only briefly summarized.<sup>10–13,37</sup> Additional details are provided in the Supplementary Information.

### Subjects

**Young monkeys.** Eighty-nine periadolescent monkeys (*Macaca mulatta*; median (s.d.) age = 2.69 (.97) years; 54% female) were phenotyped and brain imaged. This sample represents the subset of monkeys previously described by our group with complete multimodal imaging data sets (FDG-PET:  $n = 238$ ; fMRI:  $n = 107$ ).<sup>11,12,37</sup>

**Children.** Twenty-eight children (8–12 years) who met standard inclusion and exclusion criteria (see the Supplementary Information) were enrolled: 14 patients with a current pediatric anxiety disorder (mean age (s.d.) = 9.9 (1.2) years) and 14 demographically matched, psychiatrically healthy controls (mean age (s.d.) = 10.2 (1.3) years). Patients met criteria for one or more current pediatric anxiety disorders, including Generalized Anxiety Disorder, Separation Anxiety Disorder, Social Phobia or Anxiety Disorder Not Otherwise Specified. Children were recruited from local psychiatric clinics via clinician referral and from the community via media advertisements. The groups did not differ in age or sex (four females/group). Before the MRI scan, anxiety diagnoses were confirmed by a PhD-level clinical psychologist using the Kiddie-Schedule for Affective Disorders and Schizophrenia Present and Lifetime versions.<sup>38</sup> At the time of the MRI session, four patients were receiving psychotropic medications (see the Supplementary Information). Control children had no known history of mental illness (see the Supplementary Information).

### Data acquisition and processing

**Young monkeys.** T1-weighted structural and T2\*-weighted echoplanar imaging (EPI) functional MRI data were collected under anesthesia. As detailed in the Supplementary Information, EPI data were processed using standard techniques in AFNI (<http://afni.nimh.nih.gov>) except where noted otherwise. Data were processed to attenuate motion artifact, field distortions, physiological noise and slice-timing differences. To further attenuate physiological noise, average white matter and cerebrospinal

fluid time series and their temporal derivatives were residualized from the EPI time series. EPI volumes with >1 mm of motion (see the Supplementary Information) were censored from data analyses. Using this criterion, no volumes were censored for any of the young monkeys.

In a separate session, monkeys received <sup>18</sup>F-DG immediately before the 30-min No-Eye Contact challenge used to elicit the AT phenotype<sup>10–13</sup> (median (s.d.) intersession interval = 21.0 (42.4) days). Subjects were placed in a standard testing cage. An experimenter entered the room and stood motionless ~2.5 m from the subject while presenting his profile and avoiding direct eye contact. Subjects were allowed to freely respond to this ethologically relevant potential threat, similar to procedures used to assay dispositional anxiety and behavioral inhibition in children.<sup>2–3</sup> Increases in freezing and decreases in vocalizations were quantified by an experienced observer. Following behavioral testing, plasma was collected for quantifying cortisol and subjects were anesthetized and positioned in the PET scanner. Higher FDG-PET signals reflect greater regional metabolism (that is, increased FDG uptake) during the preceding No-Eye Contact challenge. FDG-PET, which provides a measure of regional brain metabolism integrated over the entire 30-min behavioral challenge, is ideally suited for assessing trait-like neural activity. AT was computed as the mean of standardized plasma cortisol, freezing and reverse-scored vocalizations in response to the human intruder's profile (30-min).

**Children.** Before scanning, children completed a mock MRI session, which has been shown to improve data quality in pediatric neuroimaging applications.<sup>39</sup> MRI data were collected using a standard eight-channel head coil. Anatomical scans were obtained with a 3D T1-weighted, inversion-recovery, fast gradient-echo prescription (inversion time/repetition time/echo time/flip angle/number of excitations/field of view/Matrix/Bandwidth: 450 ms/8.16 ms/3.18 ms/12°/1/256 mm/256 × 256/31.25 kHz) with whole-brain coverage (156 slices over 156 mm). Functional scans were obtained using a 2D T2\*-weighted EPI prescription (inversion time/repetition time/echo time/flip angle/number of excitations/field of view/Matrix: 2000 ms/25 ms/60°/240 mm/64 × 64; 40 × 4.0-mm sagittal slices; gap: 0 mm; 180 volumes). Subjects were instructed to remain motionless while remaining relaxed and awake.

Except where noted otherwise, procedures were identical to those employed in the young nonhuman primate sample. Native-space T1 images were nonlinearly registered to the Montreal Neurological Institute probabilistic template (MNI152\_T1\_1mm\_brain; <http://fsl.fmrib.ox.ac.uk>) using FLIRT, FNIRT, and FSL.<sup>40–42</sup> Single-subject EPI data were spatially normalized using the resulting transformation matrices and re-sampled to 2-mm isotropic voxels. Normalized EPI data were spatially smoothed (6 mm full-width at half-maximum). Data sets were visually inspected to ensure adequate EPI coverage without excess susceptibility or distortion. The two groups did not significantly differ in the mean amount of motion or fraction of uncensored data,  $P > .48$ ,  $d.f. = 26$  (see the Supplementary Information).

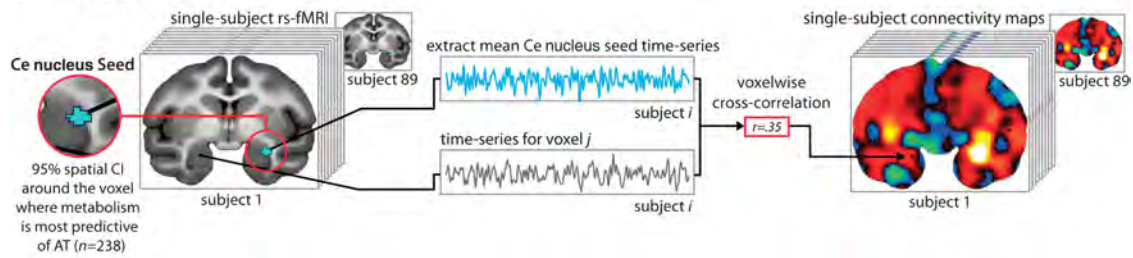
### Hypothesis testing

**Young monkeys.** A previously validated seed-based approach was used to identify the Ce nucleus functional network (see Supplementary Figure 1 and Supplementary Table 1 for additional validation analyses).<sup>37</sup> Given our focus on AT-related Ce nucleus metabolism, the seed was functionally defined as the 95% spatial confidence interval surrounding the Ce nucleus voxel, where FDG metabolism most strongly predicted AT in a superset of 238 individuals (turquoise region in Figure 1a; see Oler et al.<sup>12</sup>). Importantly, in prior work, we used *in vivo* chemoarchitectonic techniques to demonstrate that this functionally defined region corresponds to the sub-region of the primate amygdala where excitotoxic lesions attenuate anxiety,<sup>12,16,37</sup> a degree of precision that is difficult to achieve using conventional imaging techniques in humans (for example, see Herringa et al.<sup>43</sup> and Burghy et al.<sup>44</sup>). As in other recent work by our group,<sup>43,44</sup> hypothesis testing employed statistical maps that were thresholded ( $P = .05$ , corrected) based on cluster extent. Null distributions were estimated via Monte Carlo simulations (50 000 iterations; *3dClustSim*). Simulations incorporated the mean spatial smoothness of the single-subject residuals, estimated using *3dFWHMx*, and an uncorrected voxelwise threshold of  $P = 0.005$ . All hypothesis-testing analyses controlled for nuisance variation in mean-centered age and sex.

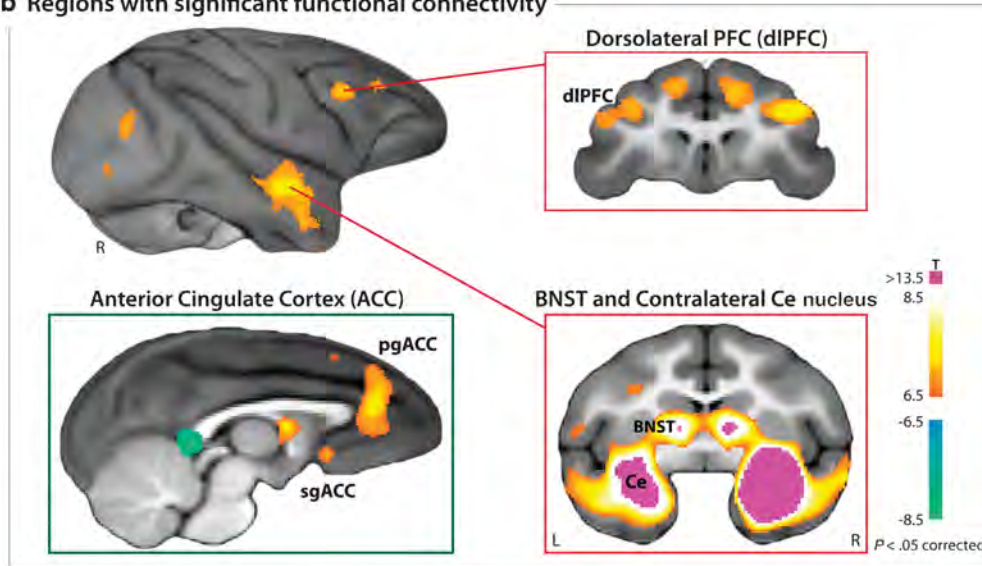
We used a standard multivariate analytic framework<sup>45,46</sup> to test whether the relationship between prefrontal-amygdalar functional connectivity and AT is statistically mediated by Ce nucleus metabolism (connectivity → metabolism → AT). As with our other analyses, we controlled for nuisance variance in mean-centered age and sex. For recent applications of this framework to neuroimaging data sets, see Shackman et al., Lim et al. and

## Intrinsic functional connectivity of the central (Ce) nucleus of the amygdala in young monkeys

### a Method overview



### b Regions with significant functional connectivity



**Figure 1.** Intrinsic functional connectivity of the central (Ce) nucleus of the amygdala in young monkeys. **(a)** Method overview. The seed for functional connectivity analyses (depicted in turquoise) was functionally defined as the 95% spatial confidence interval (CI) surrounding the Ce nucleus voxel where fluorodeoxyglucose (FDG) metabolism best predicted individual differences in anxious temperament (AT) in a superset of 238 individuals (detailed in Oler *et al.*<sup>12</sup>). Using the mean de-noised echo planar imaging (EPI) time series from the seed (see the Materials and Methods and Supplementary Information), we computed voxelwise estimates of the strength of functional connectivity for each of the 89 individuals. **(b)** Regions with significant functional connectivity with the Ce nucleus. Significant ( $P < 0.05$ , corrected) clusters included the dorsolateral prefrontal cortex (dlPFC), pregenual anterior cingulate cortex (pgACC), subgenual ACC (sgACC), bed nucleus of the stria terminalis (BNST) and contralateral Ce nucleus. Red lines indicate the locations of the two coronal slices. L, left hemisphere; R, right hemisphere.

Wager *et al.*<sup>11,47,48</sup> Satisfying the criteria of this framework would demonstrate that a significant proportion of the association between prefrontal-Ce nucleus connectivity and AT is explained by metabolism, an inference not afforded by simpler bivariate tests. Operationally, mediation required four significant tests: (a) connectivity  $\rightarrow$  metabolism, (b) connectivity  $\rightarrow$  AT, (c) metabolism  $\rightarrow$  AT and (d) controlling for variation in metabolism significantly weakens the connectivity  $\rightarrow$  AT correlation. Consistent with our prior work,<sup>11,49</sup> the final criterion was assessed using Clogg's test<sup>45,50</sup> conservatively thresholded at  $P < 0.05$  (Sidak-corrected for the total volume of the regions where connectivity predicted both Ce nucleus metabolism and AT;  $d.f. = n - 3 - \text{number of covariates}$ ). Naturally, this test does not provide evidence of causation and more complex alternative pathways cannot be rejected.

**Children.** Imaging data were processed using the same techniques employed with the nonhuman primate sample. Here, the Ce nucleus seed was anatomically defined using well-established techniques (see the Supplementary Information).<sup>37</sup> Using this seed, we tested whether children with anxiety disorders and extremely anxious young monkeys show a similar pattern of Ce nucleus functional connectivity.

## RESULTS

### Young monkeys

Whole-brain regression analyses revealed several prefrontal and subcortical regions with significant Ce nucleus functional

connectivity ( $P < 5.0 \times 10^{-9}$ , uncorrected;  $d.f. = 86$ ; Figure 1b and Supplementary Table 2). These clusters encompassed regions that project to the Ce nucleus, such as the pregenual ACC, and regions that receive projections from the Ce nucleus, such as the bed nucleus of the stria terminalis.<sup>19,32,37,51–53</sup> Other clusters encompassed regions that appear to lack direction connections with the Ce nucleus, including the dorsolateral prefrontal cortex (dlPFC) and subgenual ACC.<sup>19,32,37,51–53</sup>

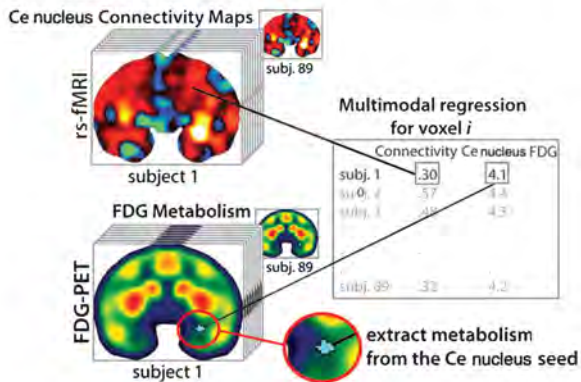
Next, we searched the entire brain for functional connections, indexed using fMRI, that are predictive of Ce nucleus metabolism, indexed using FDG-PET. This revealed that increased Ce nucleus metabolism was associated with decreased functional connectivity between the Ce nucleus and two prefrontal regions, mPFC and right dlPFC ( $P < 0.05$ , whole-brain corrected;  $d.f. = 85$ ; Figure 2 and Supplementary Table 3). The mPFC cluster encompassed several architecturally distinct regions, including pregenual ACC (area 32), frontopolar cortex (area 10M) and medial orbitofrontal cortex (OFC; area 14M; see Supplementary Figure 2). Together, the dlPFC-Ce nucleus and mPFC-Ce nucleus functional networks statistically explained 22% of the variance in Ce nucleus metabolism ( $F(2,84) = 5.92$ ,  $P < 0.001$ , uncorrected).

We also tested whether variation in intrinsic functional connectivity between these prefrontal regions and the Ce nucleus predicts AT. Paralleling the metabolism results (Figure 2), young

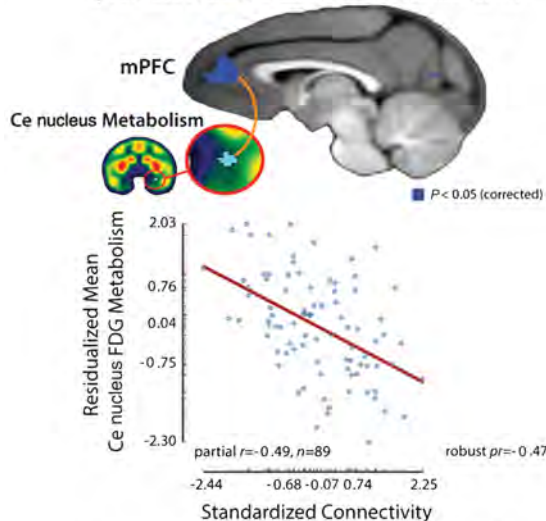
monkeys with higher levels of the anxious phenotype showed decreased functional connectivity between the Ce nucleus and both the medial and dorsolateral PFC clusters ( $P < 0.05$ , corrected;

### PFC-Ce nucleus intrinsic functional connectivity predicts Ce nucleus metabolism in young monkeys

#### a Method overview



#### b Increased connectivity with mPFC predicts reduced Ce nucleus metabolism



#### c Increased connectivity with right dIPFC predicts reduced Ce nucleus metabolism

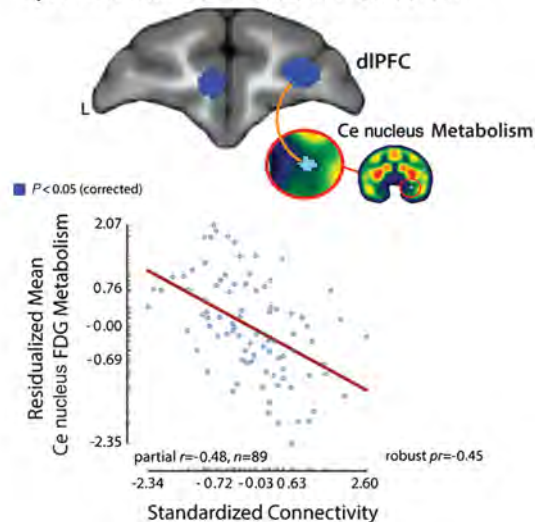


Figure 3a; Supplementary Table 4; Supplementary Figure 3). This indicates that functional connectivity measured under anesthesia is associated with objective differences in threat-elicited defensive behaviors and neuroendocrine activity assessed outside of the scanner environment. Exploratory analyses indicated that these relations were significant for each constituent of the composite AT phenotype (standardized freezing, vocalizations and cortisol: PRs =  $-0.25$  to  $-0.32$ , uncorrected  $P$ s  $< 0.03$ ;  $d.f. = 85$ ). Collectively, variation in the strength of connectivity between the Ce nucleus and these two prefrontal regions accounted for 23% of the variation in AT ( $F(2,84) = 7.29$ ,  $P < 0.001$ , uncorrected).

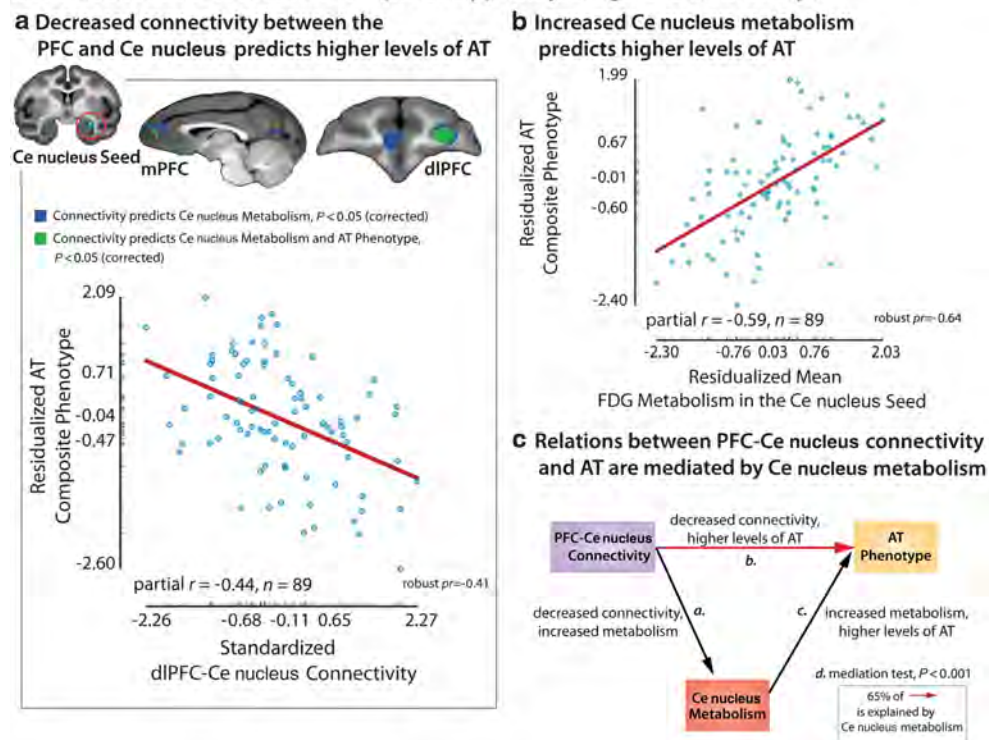
Finally, we tested whether the association between prefrontal-Ce nucleus functional connectivity and the anxious phenotype is statistically mediated by Ce nucleus metabolic activity (that is, prefrontal-Ce nucleus connectivity  $\rightarrow$  Ce nucleus metabolism  $\rightarrow$  AT), as one would expect given evidence that the Ce nucleus is a mechanistically important proximal substrate of the anxious phenotype.<sup>16</sup> We first confirmed that Ce nucleus metabolism predicts AT in the present sample ( $t = 6.70$ ,  $P = 2.3 \times 10^{-9}$ , uncorrected;  $d.f. = 85$ ; Figure 3b), consistent with our prior report.<sup>12</sup> Critically, the statistical mediation test was also significant for both the dIPFC-Ce nucleus and mPFC-Ce nucleus networks, indicating that variation in Ce nucleus metabolism explains a significant proportion of the association between prefrontal-Ce nucleus functional connectivity and AT ( $P$ s  $< .05$ , corrected;  $d.f. = 84$ ; Figure 3c and Supplementary Figure 3, Supplementary Table 4). These effects were specific; control analyses demonstrated that mediation effects were not significant for alternative network models (that is, Ce nucleus metabolism  $\rightarrow$  prefrontal-Ce nucleus connectivity  $\rightarrow$  AT;  $t$ s  $< 2.52$ ,  $P$ s  $> 0.05$ , corrected;  $d.f. = 84$ ). Taken together, these findings are consistent with the possibility that the association between prefrontal-Ce nucleus functional connectivity (that is, mPFC-Ce nucleus and dIPFC-Ce nucleus) and dispositional anxiety reflects prefrontal influences on Ce nucleus metabolism, presumably supported by polysynaptic structural pathways. Importantly, control analyses indicated that individual differences in functional connectivity, Ce nucleus metabolism and AT were not significantly associated with variation in motion artifact, uncorrected  $P$ s  $> 0.24$ ,  $d.f. = 85$  (see the Supplementary Information).

#### Children

Our results indicate that young monkeys with extreme AT are characterized by decreased functional connectivity between the PFC and Ce nucleus under anesthesia. Next, we tested whether a similar pattern is evident in quietly resting children with anxiety disorders. In fact, patients showed decreased functional connectivity between the dIPFC and Ce nucleus compared with healthy control subjects ( $P < 0.05$ , when corrected for the combined volume of the dIPFC and mPFC;  $d.f. = 24$ ; n.s. when corrected for the volume of the entire brain; Figure 4a,

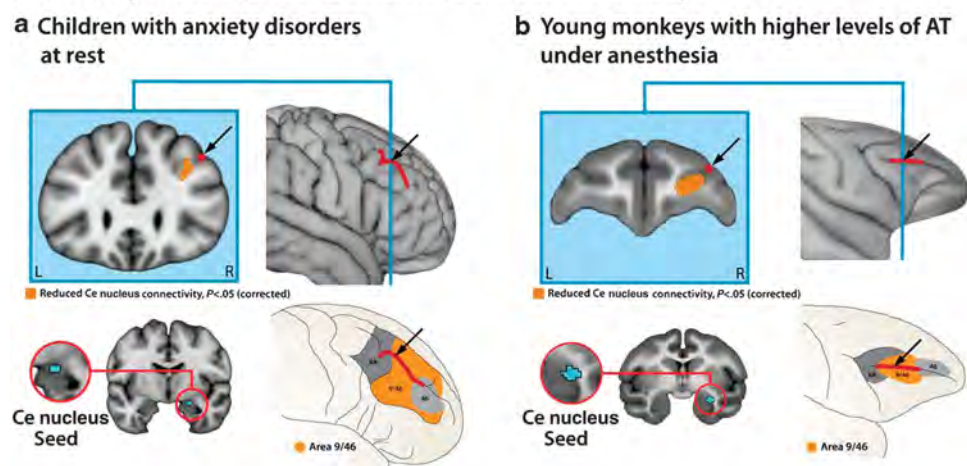
**Figure 2.** Prefrontal cortex (PFC)-central (Ce) nucleus intrinsic functional connectivity predicts Ce nucleus metabolism in young monkeys. (a) Method overview. For each monkey, a Ce nucleus functional connectivity map was computed and the mean level of fluorodeoxyglucose (FDG) metabolism was extracted from the Ce nucleus seed (turquoise region). Individual differences in Ce nucleus metabolism were used to predict voxelwise functional connectivity with the Ce nucleus throughout the brain. (b) Decreased functional connectivity with the medial PFC (mPFC) predicts increased Ce nucleus metabolism. (c) Decreased functional connectivity with the right dorsolateral PFC (dIPFC) predicts increased Ce nucleus metabolism. For illustrative purposes, scatter plots depict the partial correlations between connectivity and metabolism for the cluster averages. Axis labels indicate the minimum, maximum and inter-quartile range. Partial correlation coefficients were computed using robust regression techniques<sup>11</sup> are shown to the right of each scatter plot. PET, positron emission tomography.

### Intrinsic functional connectivity between the PFC and Ce nucleus predicts individual differences in the AT phenotype in young rhesus monkeys



**Figure 3.** Intrinsic functional connectivity between the prefrontal cortex (PFC) and central (Ce) nucleus predicts individual differences in the anxious temperament (AT) phenotype in young rhesus monkeys. **(a)** Decreased connectivity between the PFC and Ce nucleus predicts higher levels of AT. Upper panel depicts the regions within the medial PFC (mPFC) and dorsolateral PFC (dlPFC) clusters where the strength of functional connectivity significantly predicted variation in both Ce nucleus metabolism and AT (displayed in green;  $P < 0.05$ , corrected). Lower panel shows the partial correlation between dlPFC-Ce nucleus connectivity and AT (controlling for age and sex). **(b)** Increased Ce nucleus metabolism predicts higher levels of AT. **(c)** Relations between PFC-Ce nucleus connectivity and AT are significantly mediated by Ce nucleus metabolism. Summary of the four tests (*a-d*) incorporated in the multivariate mediation framework (see the Supplementary Information). For scatter plot conventions, see Figure 3. Panel depicts results for the dlPFC. Similar results were obtained for the mPFC (see Supplementary Table 4).

### Homologous dlPFC subdivisions show decreased intrinsic connectivity with the Ce nucleus in anxious children and monkeys



**Figure 4.** Homologous dorsolateral prefrontal cortex (dlPFC) subdivisions show decreased intrinsic connectivity with the central (Ce) nucleus in anxious children and monkeys. **(a)** Children with anxiety disorders at rest. Bottom-left panel shows the Ce nucleus seed (cyan in red ring). Upper-left panel depicts a coronal slice through the human dlPFC cluster (dark orange;  $P < 0.05$ , corrected for the combined volume of the mPFC and right dlPFC; n.s. when corrected for the volume of the whole brain). The intermediate frontal sulcus (IFS) is shown in dark red. Upper-right panel shows the IFS with the location of the coronal slice indicated by the blue vertical line. Bottom-right panel shows the location of the dlPFC cluster relative to the architectonic subdivisions of the human dlPFC. **(b)** Young monkeys with high levels of anxious temperament (AT) under anesthesia. Conventions are similar to **a**; dark red indicates the location of the sulcus principalis. The bottom-right panels of this figure were adapted with permission from Badre and D'Esposito.<sup>74</sup> L, left hemisphere; R, right hemisphere.

Supplementary Figures 5 and 6, and Supplementary Table 5). Significant effects were not obtained for the mPFC-Ce nucleus network in the pediatric anxiety sample. Accordingly, in the remainder of the report we focus on the evolutionarily conserved dlPFC-Ce nucleus functional network. Follow-up analyses revealed that the reduction in dlPFC-Ce nucleus connectivity evident in the pediatric patients remained significant after controlling for age and sex (Supplementary Table 5), controlling for individual differences in motion (see the Supplementary Information) or excluding four medicated patients (see the Supplementary Information). Collectively, these results demonstrate that decreased functional connectivity between the dlPFC and Ce nucleus is associated with extreme anxiety outside the imaging environment in both quietly resting children and anesthetized young monkeys (Figures 4a and b). The consistency of this relationship across species and levels of awareness at the time of scanning makes it unlikely that it reflects either a sedation artifact or differences in stress elicited by the scanner environment.

## DISCUSSION

The present results provide evidence that extreme anxiety early in life is associated with evolutionarily conserved alterations in the strength of intrinsic functional connectivity between the dlPFC and Ce nucleus. Among both children and monkeys, individuals with low levels of connectivity were characterized by high levels of anxiety when assessed outside of the scanner environment (Figures 2 and 4), raising the possibility that chronically altered functional coordination between these regions contributes to the development of pathological anxiety. In the larger sample of young monkeys, decreased functional connectivity with the dlPFC as well as the mPFC was associated with elevated metabolism in the Ce nucleus, a mechanistically important substrate for trait-like differences in anxiety.<sup>12,16</sup>

In children, AT is a well-established risk factor for the development of anxiety and other psychiatric disorders<sup>3,4</sup> and our results provide a novel framework for understanding the mechanisms underlying this liability. Prior work suggests that Ce nucleus metabolism underlies much of the experience-dependent risk that contributes to the development of extreme anxiety.<sup>12,13</sup> Likewise, recent work suggests that prefrontal-amygdalar functional connectivity can be influenced by early adversity.<sup>43,44</sup> This plasticity suggests that interventions targeting either Ce nucleus metabolism or the larger functional circuit in which it is embedded may prove to be effective strategies for reducing anxiety in children who, because of their extreme temperament, are at increased risk for developing psychopathology. By virtue of its well-established capacity for regulating mood, attention, memory and action,<sup>34,54–56</sup> the dlPFC is well positioned to translate early-life experiences into enduring alterations in Ce nucleus function.

Given the absence of established direct projections, the association between dlPFC-Ce nucleus functional connectivity and early-life anxiety is presumably supported by a polysynaptic structural network. Although our results do not identify the precise constituents of this circuit, there is growing mechanistic evidence that the PFC can influence information processing in distal brain regions via biasing signals that propagate across polysynaptic structural pathways.<sup>26,57,58</sup> This network could reflect either undiscovered projections<sup>59,60</sup> or established projections from the dlPFC to neighboring regions of the dorsal amygdala, such as the magnocellular division of the basal nucleus, that are strongly interconnected with the Ce nucleus. In particular, the dlPFC projects to a dorsal region of the basal nucleus that lies within a few millimeters of the Ce,<sup>19,51–53</sup> a difference that cannot be resolved using conventional fMRI techniques (see the Supplementary Information).

Analyses of data obtained from the larger nonhuman primate sample revealed that Ce nucleus metabolism statistically mediates

the association between prefrontal-amygdalar connectivity and AT, suggesting that Ce nucleus metabolic activity represents an intermediate link between intrinsic functional connectivity and chronically elevated anxiety (that is, decreased prefrontal-Ce nucleus connectivity → increased Ce nucleus metabolism → increased anxiety; Figure 3). Notably, this pattern was evident for functional connectivity between the Ce nucleus and two regions of the PFC: dlPFC and mPFC. Although our results do not provide evidence that the mPFC-Ce nucleus is evolutionarily conserved, this may simply reflect insufficient power in the pediatric anxiety sample. Other work highlights the potential importance of the mPFC-Ce nucleus functional network. For example, reduced functional connectivity between the mPFC and amygdala has been shown to predict heightened dispositional anxiety in several human imaging studies.<sup>59,60</sup> The present results suggest that this association between connectivity and anxiety stems from chronically elevated metabolic activity in the Ce nucleus.

Clearly, important challenges remain. As with other brain imaging studies, our analyses do not permit mechanistic inferences and alternative causal pathways are possible. Like other studies focused on measures of functional connectivity, our conclusions are tempered by questions about the origins and significance of correlated fluctuations in the blood oxygen level-dependent fMRI signal.<sup>61–63</sup> Furthermore, although it is clear from lesion studies that the Ce nucleus is mechanistically involved in AT,<sup>16</sup> we cannot reject the possibility that both increased anxiety and reduced prefrontal functional connectivity reflect symptoms of chronically elevated Ce nucleus metabolism. In particular, the Ce nucleus is well positioned to influence the signal-to-noise ratio of information processing in the PFC,<sup>64–67</sup> via projections to diffuse neuromodulatory systems in the basal forebrain and striatum. Dampened prefrontal signal-to-noise ratio would manifest as reduced functional connectivity.<sup>68</sup> A key challenge for future studies will be to use mechanistic techniques in nonhuman primates to adjudicate between these alternatives and to more fully delineate the polysynaptic circuit supporting correlated activity in the dlPFC and Ce nucleus. Combined with fMRI, transcranial magnetic stimulation represents a noninvasive alternative strategy that could be applied in humans.<sup>69,70</sup> It will also be important to test whether existing anxiolytic compounds rescue dlPFC-Ce nucleus functional connectivity. If so, then this biomarker could potentially be used to evaluate novel therapeutics.<sup>36,71</sup> Finally, it will be useful to more fully evaluate whether aberrant dlPFC-Ce nucleus functional connectivity is a transdiagnostic feature of the anxiety disorders and whether it represents a substrate for childhood AT or related constructs, such as behavioral inhibition and shyness, that were not assessed in the pediatric sample.

## CONCLUSIONS

Existing treatments for anxiety disorders are inconsistently effective or associated with significant adverse effects,<sup>5,7</sup> highlighting the need to identify and understand the neural mechanisms that confer risk. Building on prior mechanistic and neuroimaging work, the present study demonstrates that extreme anxiety is associated with reduced functional connectivity between the dlPFC and Ce nucleus in anesthetized young monkeys and quietly resting children. Translational brain imaging strategies, like that featured in the present report, provide a powerful tool for closing the gap separating the mechanistic insights afforded by nonhuman animal models from the complexity of human psychopathology and accelerating the pace of therapeutic development.<sup>35–36</sup> Our results also indicate that the association between prefrontal-amygdalar connectivity and anxiety is statistically mediated by Ce nucleus metabolism. Importantly, these results are fundamentally grounded in psychiatrically important emotional behaviors. For example, differences in the strength of prefrontal-Ce nucleus functional connectivity were found to predict anxiety-related behaviors (that is, behavioral inhibition) and cortisol elicited by an

ethologically relevant threat in freely behaving young monkeys. The use of a relatively large discovery sample, functionally and mechanistically defined seed region, well-validated primate model of pediatric anxiety and clinical confirmatory sample enhances confidence in the translational significance of these results.<sup>72</sup> More broadly, the present study provides unique evidence that early-life temperament does not just reflect differences in neural reactivity to trait-relevant cues (for example, the amygdala's threshold to respond or peak response to threat<sup>2,73</sup>), but is also embodied in the intrinsic activity of the brain.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

NHK, JAO, DSP, MJS and SES designed the study. SES collected monkey data. LEW collected human data. ALA optimized imaging methods. DSP, NHK and GMR made childhood psychiatric diagnoses. RMB and ASF developed analytical tools. RMB, JAO, AJS and ASF performed data processing for monkey data. DRM, LEW, JAO, AJS and RMB performed data processing for human data. RMB, AJS, JAO and NHK analyzed monkey data. DRM, LEW, JAO, AJS, RMB and NHK analyzed human data. AJS, NHK, RMB, ASF, JAO, RJD and DSP interpreted data. AJS, ASF, NHK, JAO, LEW and RMB wrote the paper. AJS, ASF and NHK created the figures. AJS, JAO and LEW created the tables. NHK supervised the study. All authors contributed to revising the paper.

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