Amygdala-centred functional connectivity affects daily cortisol concentrations: a putative link with anxiety

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The amygdala plays a critical role in emotion. Its functional coupling with the hippocampus and ventromedial prefrontal cortex extending to a portion of the anterior cingulate cortex (ACC) is implicated in anxiogenesis and hypothalamic-pituitary-adrenal (HPA) system regulation. However, it remains unclear how amygdala-centred functional connectivity (FC) affects anxiety and cortisol concentrations in everyday life. Here, we investigate the relationship between daily cortisol concentrations (dCOR) and amygdala-centred FC during emotional processing in forty-one healthy humans. FC analyses revealed that higher dCOR predicted strengthened amygdala-centred FC with the hippocampus and cerebellum, but inhibited FC with the supramarginal gyrus and a perigenual part of the ACC (pgACC) when processing fearful faces (vs. neutral faces). Notably, the strength of amygdala-hippocampus FC mediated the positive relationship between cortisol and anxiety, specifically when the effect of amygdala-pgACC FC, a presumptive neural indicator of emotional control, was taken into account. Individuals with diminished connectivity between the amygdala and pgACC during fear-related processing might be more vulnerable to anxiogenesis as it pertains to greater circulating cortisol levels in everyday life. Individual functional patterns of amygdala-hippocampal-pgACC connectivity might provide a key to understand the complate link between cortisol and anxiety-related behaviors.

The hypothalamic-pituitary-adrenal (HPA) axis is an endocrine system that helps the organism adjust to internal and external environmental challenges. Glucocorticoid (cortisol in humans), its end hormonal product, plays a critical role in regulating the HPA axis through its major receptors in the central nervous system (CNS): glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs are expressed throughout the brain, while MRs are more restricted. However, both exist in the hippocampus (HC) and amygdala1,2, indicating the importance of these 2 limbic structures in HPA axis regulation.

The amygdala is known to stimulate corticotropin releasing hormone (CRH)-producing neurons in the hypothalamic paraventricular nucleus (PVN) via intermediating neurons in the bed nucleus of the stria terminalis and other hypothalamic adjacent nuclei, interacting with the HC and medial prefrontal cortex (mPFC)3. Interestingly, this pathway becomes activated when an organism anticipates a potential threat arriving, but not necessarily when a salient threat actually arrives3. Indeed, previous meta-analytic functional magnetic resonance imaging (fMRI) studies have confirmed that the amygdala responds to fearful faces (that are not themselves imminent

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in healthy participants as well, especially when their cortisol levels were elevated by stress or pharmacological manipulation15, 17. Some fMRI studies have reported that greater baseline cortisol levels, measured from 10 to tens of minutes before MRI with experimental tasks, predicted attenuated amygdala responses to fearful faces (vs. happy faces)16 and oppositely heightened amygdala responses to emotional faces (vs. animals)17. Similarly, higher baseline cortisol was associated with more diminished FC between the amygdala and perigenual ACC (pgACC) during rest18, although participants’ tension and apprehension might have boosted baseline cortisol levels. In contrast, an fMRI study using saliva cortisol collected at home on non-scanning days has found that greater (normative) diurnal cortisol decline showed diminished amygdala activity and enhanced vmPFC activity when participants attempted to allay their negative affect19. However, no study has so far probed how task-irrelevant unstimulated cortisol affects amygdala-centred FC during emotional processing, and more specifically how FC between the amygdala, HC, and vmPFC is involved in the relationship between cortisol and anxiety/depression.

In contrast to externally stimulated cortisol, the literature investigating crosstalk between endogenous cortisol and the amygdala and its neural connectivity is surprisingly limited, notwithstanding the evidence indicating that unstimulated cortisol is a key neurobiological component of emotional dysregulations associated with anxiety and depression20. Some fMRI studies have reported that greater baseline cortisol levels, measured from 10 to tens of minutes before MRI with experimental tasks, predicted attenuated amygdala responses to fearful faces (vs. happy faces)16 and oppositely heightened amygdala responses to emotional faces (vs. animals)17. Similarly, higher baseline cortisol was associated with more diminished FC between the amygdala and perigenual ACC (pgACC) during rest18, although participants’ tension and apprehension might have boosted baseline cortisol levels. In contrast, an fMRI study using saliva cortisol collected at home on non-scanning days has found that greater (normative) diurnal cortisol decline showed diminished amygdala activity and enhanced vmPFC activity when participants attempted to allay their negative affect19. However, no study has so far probed how task-irrelevant unstimulated cortisol affects amygdala-centred FC during emotional processing, and more specifically how FC between the amygdala, HC, and vmPFC is involved in the relationship between cortisol and anxiety/depression.

We therefore investigated the effect of daily circulating cortisol levels on amygdala-centred FC during an emotional faces perception task using saliva gathered in task-irrelevant contexts for 2 consecutive typical weekdays, which provides reliable estimates of baseline cortisol levels21. We hypothesized that, while processing fearful faces (vs. neutral faces), FC between the amygdala, HC, and vmPFC would be significantly associated with task-irrelevant cortisol as well as anxiety/depression. Provided that amygdala-HC-vmPFC FC was significantly correlated with both cortisol and anxiety/depression, we further examined a possible mediation effect of the amygdala-HC-vmPFC FC on the relationship between cortisol and anxiety/depression.

Table 1. Salivary cortisol data (nmol/L). Abbreviations: SD, standard deviation; dCOR, daily cortisol concentrations; AUCg, area under the curve with respect to ground (at 3 points); CAR, cortisol awakening response (i.e., area under the curve with respect to increase); DS, diurnal slope.

|                | Mean   | SD     |
|----------------|--------|--------|
| Time 1 (awakening) | 14.90  | 9.55   |
| Time 2 (30 min after awakening) | 24.45  | 10.81  |
| Time 3 (bedtime)   | 4.15   | 4.96   |
| dCOR             | 43.51  | 22.47  |
| AUCg             | 259.94 | 137.77 |
| CAR              | 2.39   | 2.00   |
| DS               | 10.75  | 6.62   |

Results

Cortisol data. Salivary cortisol data is shown in Table 1. With the exception of DS, all cortisol measures failed the Shapiro-Wilk test and, consequently, were square root transformed. No cortisol measures were significantly associated with age, sex, menstrual period, BMI, monthly alcohol consumption, years of education, sleep duration, sleep quality, perceived stress, or time of awakening during the 2 saliva collection days (see Supplementary material and Table S1). Due to the great commonality between dCOR and AUCg (Table S1; r = 0.96, p < 0.001) and their overlapping results, we focused on dCOR in the following analyses.

Amygdala-centred FC during fearful faces processing. FC results for the simple effect of fearful faces in the entire sample are presented in Fig. 1 for each side of the amygdala seed. Both R-Amy and L-Amy showed increased HC connectivity (Table 2).

Whole-brain analysis. Whole-brain analyses revealed that higher dCOR levels predicted significantly increased R-Amy-centred FC with the left HC (T = 4.75, FDR-corrected p = 0.004) and the left cerebellum (T = 3.55, FDR-corrected p = 0.047), and decreased FC with an anterior part of the left supramarginal gyrus (SMG) (T = −3.72, FDR-corrected p = 0.043) upon the appearance of fearful faces (vs. neutral faces). No other FC analyses showed significant relationships with cortisol indices other than CAR. The results of CAR are shown in Supplementary Material.
**Figure 1.** Amygdala-centred functional connectivity during fearful faces processing. Seed region: the amygdala that showed significantly increased activation in response to fearful faces (vs. neutral faces) in this sample (L-Amy: MNI $-21 -10 -17$, $351 \text{mm}^3$, R-Amy: MNI $21 -10 -17$, $189 \text{mm}^3$), which are comprised of the caudomedial portion of the amygdala. Clusters were significant at FDR-corrected $p < 0.05$ [both height threshold and cluster threshold (cluster-size correction)]. Abbreviations: L-Amy, left amygdala; R-Amy, right amygdala; MNI, Montreal Neurological Institute space; FDR, false-discovery rate.

| Region                                | Side | Voxels | MNI Coordinate | $T$  |
|---------------------------------------|------|--------|----------------|------|
| Right                                 |      |        |                |      |
| Parahippocampal region (including a part of the amygdala and the hippocampus) | R    | 441    | $20 -8 -16$    | 17.77|
| Hippocampus                           | L    | 119    | $-20 -8 -18$   | 6.98 |
| Middle temporal gyrus                 | L    | 47     | $-70 -52 -2$   | 5.85 |
| Subcallosal gyrus                     | L    | 29     | $-2 12 -10$    | 6.41 |
| Left                                  |      |        |                |      |
| Parahippocampal region (including a part of the amygdala, hippocampus, putamen, and pallidum) | L    | 648    | $-16 -6 -14$   | 22.30|
| Occipital fusiform gyrus (including a part of the cerebellum) | R    | 100    | $18 -4 -18$    | 6.58 |
| Parahippocampal region (including a part of the amygdala and hippocampus) | R    | 88     | $20 -76 -18$   | 6.27 |
| Putamen                               | R    | 60     | $28 8 -6$      | 6.26 |

**Table 2.** Amygdala-centred functional connectivity during fearful faces processing Seed region is the amygdala whose cluster showed significantly increased activation in response to fearful faces (vs. neutral faces): L-Amy: MNI $-21 -10 -17$, $351 \text{mm}^3$, R-Amy: MNI $21 -10 -17$, $189 \text{mm}^3$. These clusters are comprised of the caudomedial portion of the amygdala. Significant at whole-brain FDR-corrected $p < 0.05$. 
Seed-to-voxel analysis. Seed-to-voxel analysis further specified that 2 different clusters within the HC were more strongly connected with R-Amy as a function of dCOR: 1 was located on its anterior part [anterior HC (aHC): MNI $-$30 $-$22 $-$16, 280 mm$^3$, $T = 5.41$, $p < 0.001$] (Fig. 2a,b) and the other was on its posterior part [posterior HC (pHC): MNI $-$30 $-$38 $-$4, 144 mm$^3$, $T = 4.30$, $p < 0.001$] (Fig. 2a,c). A scatter plot of these clusters is presented in Fig. 2d.

Furthermore, we found significant FC between the R-Amy and pgACC in the seed-to-voxel analysis (MNI 0 30 14, 120 mm$^3$, $T = 4.40$, $p < 0.001$) (Fig. 3a), although it was not found in the whole-brain analysis possibly because the ROI of vmPFC was too extensive and functionally heterogeneous to detect a significance of $\beta$ values averaged across all voxels therein. The scatter plot of the pgACC is presented in Fig. 3b. Thus, the amygdala-pACC FC was factored into subsequent analyses.

Regression analysis: amygdala-centred FC and anxiety/depression. Step-wise regression analyses demonstrated that R-Amy–pHC FC was the sole significant predictor for somatization symptoms [$R^2 = 0.10$, $F(1,39) = 4.16$, $p = 0.049$, $\beta = 0.31$] and anxiety symptoms [$R^2 = 0.15$, $F(1,39) = 6.75$, $p = 0.013$, $\beta = 0.38$], respectively. No other amygdala-centred FC was significantly associated with any HSCL symptom.

Mediation analysis: effect of amygdala-centred FC on the relationship between cortisol and anxiety/depression. Due to amygdala-pHC FC’s significant correlation with both cortisol and anxiety, we examined whether it would mediate the relationship between anxiety symptoms and dCOR. At first, the simple correlation coefficients between anxiety (X) and dCOR (Y) were not significant ($r = 0.26$, $p = 0.102$). However, a significant direct effect of anxiety symptoms on dCOR emerged when the effect of amygdala-pACC FC was factored in as a covariate [unstandardized regression coefficient ($B$) = 0.07, $p = 0.040$]. Furthermore, when amygdala-pHC FC was assigned as M, we observed a significant indirect effect of amygdala-pHC FC on the relationship between dCOR and anxiety symptoms [$B = 0.05$, 95% CI = 0.00–0.12; standardized regression coefficient ($\beta$) = 0.20, 95% CI = 0.00–0.50; and $p = 0.040$] (Fig. 4). The statistical significance of the direct effect disappeared...
in the mediation model \(B = 0.03, p = 0.40\), indicating complete mediation by the amygdala-pHC FC. The mediation effect accounted for 61.4% of the anxiety symptoms’ total effect on dCOR.

**Discussion**

We hypothesized that FC between the amygdala, HC, and vmPFC in emotional processing would be significantly associated with task-irrelevant cortisol. Supporting this hypothesis, the results revealed that greater dCOR was correlated with increased amygdala-centred FC with the left HC and diminished FC with the pgACC in responses to fearful faces (vs. neutral faces). Additionally, exploratory whole-brain analysis found that the cerebellum strengthened FC with the amygdala, whereas the SMG decreased it during processing fearful faces (vs. neutral faces). Especially, the SMG has been reported to have lower FC with the amygdala in anxiety disorder patients^{22–25}, although its exact function has yet to be elucidated. The present study is the first to show that unstimulated basal cortisol levels, measured task-irrelevantly in everyday life, is associated with the strength of FC between the amygdala, HC, and pgACC that are implicated in both anxiety and HPA axis regulation\(^3,7\). Given that even single basal cortisol levels measurements have been reported to be significantly correlated with total cortisol output\(^8\), dCOR might reflect a similar component of total cortisol output that is more stable over time than DS or CAR\(^7\), which would make it a potent predictor for the strength of neural connectivity between HPA-axis regulatory regions, specifically between the amygdala, HC, and pgACC.
Interestingly, the present study further demonstrated a significant mediation effect of amygdala-pHC FC on the positive relationship between dCOR and anxiety symptoms, provided that the effect of amygdala-pgACC FC was factored in as a covariate (although the pgACC itself was not significantly associated with anxiety directly). The pgACC has been implicated in emotional regulation and its perturbed functional coupling with the amygdala has been substantiated in anxiety disorders. Since the pgACC has been reported to have a regulatory effect on the amygdala responses to emotional stimuli, individuals with lesser connectivity between the amygdala and pgACC during fear-related processing might be susceptible to anxiogenesis as it pertains to greater dCOR. Complicated interactions between the amygdala, HC, and pgACC might contribute to the controversy over the link between cortisol and anxiety disorders.

The R-Amy cluster used as a seed in this study was located on the amygdala's caudomedial portion, which encompasses its medial nucleus (MeA) and central nucleus (CeA), both of which are involved in stimulating the complicate link between cortisol and anxiety-related behaviors. Future studies are needed to elucidate the functional patterns of the amygdala-hippocampal-pgACC neural circuit might provide a key to better understand into account. Individuals with lesser connectivity between the amygdala and pgACC during fear-related processing might be susceptible to anxiogenesis as it pertains to greater dCOR. Complicated interactions between the amygdala, HC, and pgACC might contribute to the controversy over the link between cortisol and anxiety disorders.

At the level of the CeA and MeA nuclei, the R-Amy was connected with the posterior and anterior parts of the left HC in proportion to dCOR levels, which suggests the importance of contralateral interaction between the R-Amy and the left HC when anticipating a possible threat. Substantial evidence indicates that the R-Amy is more important in emotional face perception. A diffusion-tensor tractography study indicated that a bundle of fibers originating on 1 side of the amygdala can project to the contralateral side, extending to contralateral adjacent regions such as the HC. An anterior part of the left HC is associated with face discrimination and fear-specific emotional processing, and a posterior part of the left HC is generally involved in visual perception processing. In contrast, anterior and posterior portions of the right HC are both linked with a wider range of emotions (not-specific to fear). Altogether, these findings suggest that the interaction between the R-Amy and left HC might be especially important in fear-related emotional processing and that dCOR might be highly relevant to this interaction.

Besides dCOR, we found a significant effect of CAR on decreased I-Amy FC to the parahippocampal gyrus and right temporal pole (as shown in Supplementary material). No past study has found a significant association of CAR with local activation or FC originated from the amygdala, notwithstanding the assumed relationship between them. In contrast, we did not find a significant effect of DS on amygdala-centred FC, despite the report that normative diurnal decline was associated with diminished amygdala activity and enhanced mPFC activity in older adults. The diurnal cortisol variation in young adults might be different from that in older adults who are susceptible to its perturbation due to aging. Additionally, a resting-state fMRI study found a significant positive correlation between pre-scan cortisol levels and diminished FC between the amygdala and pgACC, partly conforming with the current findings on the correlation between greater dCOR and diminished amygdala-pgACC FC. Unstimulated baseline cortisol levels might be a steady biomarker for neural connectivity strength between the amygdala and the pgACC as well as HC.

This study's limitations should be considered when interpreting the results. First, we used cortisol measurements from 3 time points for 2 consecutive days. Particularly, CAR was measured by 2 points sampling, but not 3 points (or more) sampling as recommended. The results of CAR were thus reported as additional information. Second, we did not use an objective method of verifying awakening time (e.g., actigraphy or electrocardiography) or verifying sampling times (e.g., an electronic monitoring system), although a previous study using polysomnography found no differences between self-reported awakening times and objectively recorded awakening times. However, these 2 limitations are unlikely to considerably undermine the results of dCOR, which is relatively unaffected by awakening-related factors as compared to CAR. Third, the sample is relatively small, although it was still reasonably large compared to previous studies focusing on unstimulated cortisol. Finally, although the assessment of anxiety symptoms preceded MRI scans and saliva collection in this study, the causality between amygdala-centred connectivity, cortisol, and anxiety remains to be unraveled. Future studies examining larger samples with longitudinal measurement and different age tiers will further delineate the precise chronological relationships between cortisol, anxiety, and amygdala-HC-pgACC connectivity and the complicated mechanism behind the relationship between cortisol and emotional dysregulation.

In conclusion, we provide the first evidence that daily cortisol concentrations, measured task-irrelevantly in everyday life, is associated with the strength of amygdala-centred FC with the HC and pgACC that are implicated in anxiogenesis and HPA axis regulation. Notably, FC strength with the left HC at the seed of the central and medial nuclei of the right amygdala has a strong correlation with daily cortisol concentrations. Furthermore, amygdala-HC FC mediated the relationship between cortisol and anxiety, provided that the effect of amygdala-pgACC FC strength, a presumable neural indicator of individual emotional regulation, was taken into account. Individuals with lesser connectivity between the amygdala and pgACC during fear-related processing might be more vulnerable to anxiogenesis as it pertains to greater circulating cortisol secretion. Individual functional patterns of the amygdala-hippocampal-pgACC neural circuit might provide a key to better understand the complicate link between cortisol and anxiety-related behaviors. Future studies are needed to elucidate the mechanism behind the relationship between cortisol, anxiety, and amygdala-HC-pgACC neural circuit.
Methods

Participants. The Kitasato University Hospital Institutional Review Board approved the study (approval number: C11–690). All experimental methods were carried out in accordance with the ethical guidelines determined by the National Ministry of Health, Labour and Welfare and the Declaration of Helsinki. All participants provided written informed consent. Forty-one healthy participants were recruited via advertisements in a local magazine and billboards at Kitasato University (all right-handed, 25 women, mean age = 21.9 years, range = 20–33, standard deviation (SD) = 2.9). The eligibility criteria were (1) right-handedness as defined by the Edinburgh Handedness Inventory47, (2) no Axis-I psychiatric disorders or substance-abuse history as determined using the Mini-International Neuropsychiatric Interview48, (3) no major medical illnesses, (4) no regular intake of psychotropics, steroids, or opioids, (5) no head injury with loss of consciousness, and (6) no history of habitual smoking. No one was engaged in shift work or had jet lag. The participants overlapped with those of our previous study examining a cognitive measure's neural correlates33. In this study, however, we focused on completely different hypotheses, and the analyses reported here do not overlap with those previously published.

Study procedures. All participants completed MRI scans and saliva collection, which were generally conducted within 2 weeks after the initial assessment. Thirty-two percent of them gathered their saliva prior to MRI scans (average interval between saliva collection and MRI scans ± SD = 3.8 ± 13.8 days). At the initial assessment, data were obtained concerning age, sex, body mass index (BMI), monthly alcohol consumption, and years of education in order to account for their possible confounding effects on cortisol46. Women were asked to disclose the presence of menstrual irregularity, their typical menstrual period, and last menstruation date. None used oral contraceptives. The scoring methods for BMI, monthly alcohol consumption, and menstrual period are detailed in the Supplementary material.

Psychological assessment. Psychological distress including anxiety and depression was assessed at the first assessment using the Hopkins symptom checklist (HSCL)49, which is reported to be closely associated with HPA axis function50. We used the Japanese version of HSCL, whose reliability and validity have been established51, 52. The HSCL is a self-report questionnaire measuring distress experienced during the past week across five dimensions: (1) somatization, (2) obsessive–compulsive behavior, (3) interpersonal sensitivity, (4) anxiety, and (5) depression. These symptoms were rated for 54 items on a 4-point frequency scale (i.e., from 1: “Not-at-all” to 4: “Frequently”). Mean and SD of each subscale is shown in Table S2.

Saliva collection and cortisol assay. Salivary cortisol was measured 3 times daily: upon awakening (T1), 30 min after awakening (T2), and at bedtime (T3). At the first meeting, an experimenter fully explained the procedures at each time point and demonstrated saliva collection. Participants were encouraged to practice taking their saliva using a sample tube and to read a collection protocol at home. Participants collected saliva on 2 consecutive typical weekdays using personalised kits with 6 tubes containing Salimetric Oral Swabs (Salimetrics, Inc., State College, PA), each labeled in a unique colour with date and time of measurement (e.g., The 1st day, at bedtime). Participants were allowed to decide which day they would start saliva collection provided it was a typical weekday within 2 weeks after the initial assessment.

During the consecutive 2 days and 1 night before starting, alcohol was strictly prohibited. Firm restrictions also applied to consuming any food or drink besides water, exercising, tooth-brushing, and showering or bathing within 30 min after awakening or 1 h before bedtime. At awakening, participants were required to record the time they went to bed and awoke and to rate sleep quality and perceived stress on a 4-point scale (i.e., 0–3). Within 24 h after completing saliva collection, the refrigerated samples were transported to the National Institute of Occupational Safety and Health.

For the salivary assay, slowly thawed samples were centrifuged at 3000 rpm (G-force = 1710) for 15 min. Salivary cortisol concentrations were determined using enzyme-linked immunoassay kits (IBL International, Hamburg, Germany). Inter-assay and intra-assay concentration variations were below 7.3% and 9.3%, respectively. Because the heterogeneity of cortisol measures prevented previous findings from converging, we used the 3 major cortisol indices: cortisol awakening response (CAR), diurnal slope (DS), and daily cortisol concentrations (dCOR). As for CAR, however, the findings were reported in Supplementary material because 3 (or more) time points sampling is recommended for the assessment of CAR45. Based on the previous formula49, these 3 measures were calculated as follows: [(T1 + T2) × 0.5 h / 2] – [T1 × 0.5 h] for CAR (i.e., the area under the curve with respect to increase), (T3 – T1) for DS, and the sum of 3 time points for dCOR. Additionally, the area under the curve with respect to ground (AUCg) was reported as a reference index of dCOR: 

\[
\text{AUCg} = \left(\frac{\text{T1}_+ \text{T2}}{2}\right) - \left(\frac{\text{T1}}{2}\right)
\]

Each measure was averaged across the 2 collection days. The cortisol measures were square-root transformed if the Shapiro-Wilk test indicated that they were not normally distributed.

fMRI experimental design. The emotional faces perception task was a modified version of the face-house matching task34 (Fig. 5). The task procedures were described elsewhere35. Briefly, after a 2000-ms visual display consisting of 4 empty frames in vertically- or horizontally-paired positions with either the 2 horizontal or the 2 vertical frames being highlighted, a fixation point was presented at the screen’s center for 1000 ms. Next, a pair of faces and a pair of houses were presented at the frames’ locations for 250 ms. Paired houses were always preceded by the 2 highlighted frames, and participants were instructed to attend to these highlighted frames and subsequent houses. Fearful faces that emerge outside the focus of attention, as compared to neutral faces, are known to reliably elicit responses of the amygdala36. The positions of the paired faces or houses (vertical or horizontal) and the types of facial expressions (neutral or fearful) were presented in a random order for each subject. The stimulus intervals were jittered between 3500 ms and 12500 ms for the 48 trials with a mean of 7500 ms. The task duration was 8 min 51 sec (including dummy scans).
fMRI acquisition. Anatomical and functional MRI scans were acquired using a 1.5-tesla GE Signa scanner (Signa HDxt; GE Healthcare, Wauwatosa, WI) with an 8-channel phased-array head coil. Structural images were acquired with a 3D T1-weighted sequence (slice thickness without slice gap = 1.2 mm, field of view (FOV) = 240 mm, matrix = 288 × 256, repetition time (TR) = 13.5 ms, echo time (TE) = 5.8 ms, and flip angle (FA) = 20°). For functional images, data were acquired using fast-gradient-echo-planar T2*-weighted imaging with 5 dummy volumes at the beginning of the session. Each functional volume consisted of 30–34 transverse slices (slice thickness = 4.0 mm, slice gap = 1.0 mm, FOV = 260 mm, matrix = 128 × 128, TR = 3,000 ms, TE = 40 ms, and FA = 90°).

fMRI preprocessing. We used CONN Functional Connectivity Toolbox version 16a\textsuperscript{56}; http://www.nitrc.org/projects/conn\textsuperscript{57} that was implemented in SPM12 (Institute of Neurology, University College London, London, UK; http://www.fil.ion.ucl.ac.uk/spm/software/spm12) for preprocessing and FC analyses. Preprocessing procedures consisted of: segmentation, slice-time correction, coregistration, spatial normalization to the Montreal Neurological Institute (MNI) space, Gaussian spatial smoothing (full width at half maximum (FWHM) = 8 mm). Exploratory whole-brain analysis.

Definition of the amygdala as seed region. We set the amygdala as a seed region. The seed was extracted from clusters showing significant responses to fearful faces (vs. neutral faces) \{left amygdala (L-Amy) = −21 −10 −17, 351 mm\textsuperscript{3}, right amygdala (R-Amy) = 21 −10 −17; 189 mm\textsuperscript{3}\; both significant at \(p < 0.001\) (uncorrected) within the amygdala mask defined by the Wake Forest University (WFU) PickAtlas\textsuperscript{38}\} \textsuperscript{39} (the left amygdala cluster was also significant at FWE-corrected \(p < 0.05\) with small volume correction).

Exploratory whole-brain analysis. Using these amygdala seeds, we first performed a whole-brain analysis to explore a possible association between cortisol indices and different brain regions including the HC and vmPFC. These target brain regions were defined according to a default atlas in CONN \{132 regions of interest (ROIs)\} that comprised cortical and subcortical areas in the FSL Harvard-Oxford atlas (http://www.cma.mgh.harvard.edu/fsl_atlas.html) and cerebellar areas in the Automated Anatomical Labeling (AAL) atlas (http://www.gin.cnrs.fr/AAL). The time series of blood-oxygen-level dependent (BOLD) signal values were averaged across all voxels within each target region. Bivariate-regression analyses were conducted to determine the linear association of the BOLD time series between the amygdala seed and target regions, creating FC maps for each participant. Each cortisol index was entered into a general linear model as an independent variable explaining the FC as a dependent variable. Statistical significance was defined as \(p < 0.05\) (2-sided), with false-discovery rate (FDR) corrections for multiple tests.

Seed-to-voxel analysis. When the brain regions were observed to be significantly connected with the amygdala in the whole-brain analysis, we conducted a seed-to-voxel analysis to clarify the connected regions’ locations. Due to the above-mentioned hypothesis, we adopted clusters found in the HC and vmPFC within anatomical masks of the HC, mOFC, and ACC in the WFU PickAtlas. Height threshold was set at \(p < 0.001\) with extent threshold \(k > 15\) voxels.
Regression analysis: amygdala-centred FC and anxiety/depression. Further, regression analyses were performed to examine the effect of anxious and depressive mental states (as measured by HSCL) on the amygdala-centred FC. The estimated values (βs) of amygdala-centred FC with the HC, vmPFC or the other brain regions that were shown to be significant in the whole-brain analysis (whose clusters were specified by the seed-to-voxel analysis) were extracted with MarsBar 0.43 (http://marsbar.sourceforge.net/). These amygdala-centred FC values were fed into a step-wise regression analysis with each of the 5 HSCL subscales as a dependent variable using SPSS version 24.0 (IBM, Inc., Tokyo, Japan). Statistical significance was defined as p < 0.05 (2-tailed).

Mediation analysis: an effect of amygdala-centred FC on the relationship between cortisol and anxiety/depression. Provided that amygdala-centred FC with the HC and vmPFC were significantly correlated with both cortisol and anxiety/depression, we further investigated whether the amygdala-centred FC would mediate the relationship between cortisol and anxiety/depression using PROCESS implemented in SPSS (http://processmacro.org/index.html). We estimated the bootstrapped (5,000 samples) 95% confidence intervals for the indirect effect. Statistical significance was defined as p < 0.05. Since psychological assessments preceded MRI acquisition and saliva collection, the independent variable was anxiety/depression (independent variable = X), and the dependent variable was cortisol (dependent variable = Y). Amygdala-centred FCs were assigned as the mediator (M). Additionally, when both an amygdala-HC FC and amygdala-vmPFC FC were associated with cortisol, but only 1 was correlated with anxiety/depression, the anxiety/depression-correlated FC was entered in the mediation model as M. The other (anxiety/depression-uncorrelated) FC could be incorporated as a covariate to improve the variance percentage explained for Y, because amygdala-HC FC and amygdala-vmPFC FC are presumed to function antagonistically to each other.26,31 A mediation effects’ presence was considered confirmed if (1) we would observe a statistically significant indirect effect of the variable X on Y through the mediator M, and (2) the direct effect would be reduced or nullified (i.e., partial or complete mediation, respectively) in the mediation model (as compared to the direct effect in the initial model excluding M).

Data availability statement. The data that support the findings of this study are available on request from the corresponding author [Y.H.], as long as the data sharing complies with the Japanese Ministry of Health, Labor and Welfare Ethical Guidelines. The data are not publicly available due to them containing information that could compromise research participant consent.

References
1. Reul, J. M. & de Kloet, E. R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505–11 (1985).
2. de Kloet, E. R. & Reul, J. M. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12, 83–105 (1987).
3. Herman, J. P. et al. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Compr Physiol 6, 603–21 (2016).
4. Phan, K., Wager, T., Taylor, S. & Liberzon, I. Functional neuroanatomy of emotion: a meta-analysis of emotion activation studies in PET and fMRI. NeuroImage 16, 331–48 (2002).
5. Sabatinelli, D. et al. Emotional perception: meta-analyses of face and natural scene processing. NeuroImage 54, 2524–33 (2011).
6. Fusar-Poli, P. et al. Functional atlas of emotional faces processing: a voxel-based meta-analysis of 105 functional magnetic resonance imaging studies. J Psychiatry Neurosci 34, 418–32 (2009).
7. Elkin, A. & Wager, T. D. Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am J Psychiatry 164, 1476–88 (2007).
8. Hamilton, J. P. et al. Functional neuroimaging of major depressive disorder: a meta-analysis and new integration of base line activation and neuro response data. Am J Psychiatry 169, 693–703 (2012).
9. Klimes-Dougan, B. et al. Multilevel assessment of the neurobiological threat system in depressed adolescents: interplay between the limbic system and hypothalamic-pituitary-adrenal axis. Dev Psychopathol 26, 1321–35 (2014).
10. Henckens, M. J. et al. Interindividual differences in stress sensitivity: basal and stress-induced cortisol levels differentially predict neural processing under stress. Soc Cogn Affect Neurosci 11, 663–73 (2016).
11. Merz, C. J., Stark, R., Vaitl, D., Tabbert, K. & Wolf, O. T. Stress hormones are associated with the neuronal correlates of instructed fear conditioning. Biol Psychol 92, 82–9 (2013).
12. Schmidt, A. et al. Acute effects of heroin on negative emotional processing: relation of amygdala activity and stress-related responses. Biol Psychiatry 76, 289–96 (2014).
13. Henckens, M. J., van Wingen, G. A., Joëls, M. & Fernández, G. Time-dependent effects of corticosteroids on human amygdala processing. J Neurosci 30, 12725–32 (2010).
14. Henckens, M. J., van Wingen, G. A., Joëls, M. & Fernández, G. Time-dependent effects of cortisol on selective attention and emotional interference: a functional MRI study. Front Integr Neurosci 6, 66 (2012).
15. Kiem, S. A. et al. Resting state functional MRI connectivity predicts hypothalamus-pituitary-axis status in healthy males. Psychoneuroendocrinology 38, 1338–48 (2013).
16. Vaisveser, S. et al. Neural traces of stress: cortisol related sustained enhancement of amygdala-hippocampal functional connectivity. Front Hum Neurosci 7, 313 (2013).
17. Kirkanski, K., LeMoult, J., Ordaz, S. & Gotlib, I. H. Investigating the nature of co-occurring depression and anxiety: Comparing diagnostic and dimensional research approaches. J Affect Disord 216, 123–35 (2017).
18. Weldon, A. L. et al. Stress Response to the Functional Magnetic Resonance Imaging Environment in Healthy Adults Relates to the Degree of Limbic Reactivity during Emotion Processing. Neuropsychobiology 71, 85–96 (2015).
19. Veer, I. M. et al. Endogenous cortisol is associated with functional connectivity between the amygdala and medial prefrontal cortex. Psychoneuroendocrinology 37, 1039–47 (2012).
20. Urry, H. L. et al. Amygdala and ventromedial prefrontal cortex are inversely coupled during regulation of negative affect and predict the diurnal pattern of cortisol secretion among older adults. J Neurosci 26, 4415–25 (2006).
21. Poll, E. M. et al. Saliva collection method affects predictability of serum cortisol. Clin Chim Acta 382, 15–9 (2007).
22. Makovac, E. et al. Alterations in Amygdala-Prefrontal Functional Connectivity Account for Excessive Worry and Autonomic Dysfunction in Generalized Anxiety Disorder. Biol Psychiatry 80, 786–795 (2016).
23. Prater, K. E., Hosanagar, A., Klumpp, H., Angstadt, M. & Phan, K. L. Aberrant amygdala-frontal cortex connectivity during perception of fearful faces and at rest in generalized social anxiety disorder. Depress Anxiety 30, 234–41 (2013).
Hahn, A. et al. Reduced resting-state functional connectivity between amygdala and orbitofrontal cortex in social anxiety disorder. *Neuroimage* **56**, 881–9 (2011).

Liao, W. et al. Altered effective connectivity network of the amygdala in social anxiety disorder: a resting-state fMRI study. *PLoS One* **5**, e15238 (2010).

Edwards, S., Clow, A., Evans, P. & Hucklebridge, F. Exploration of the awakening cortisol response in relation to diurnal cortisol secretory activity. *Life Sci* **68**, 2093–103 (2001).

Ross, K. M., Murphy, M. L., Adam, E. K., Chen, E. & Miller, G. E. How stable are diurnal cortisol activity indices in healthy individuals? Evidence from three multi-wave studies. *Psychoneuroendocrinology* **39**, 184–93 (2014).

Schiller, D. & Delgado, M. R. Overlapping neural systems mediating extinction, reversal and regulation of fear. *Trends Cogn Sci* **14**, 268–76 (2010).

Shin, L. & Liberzon, I. The neurocircuity of fear, stress, and anxiety disorders. *Neuropsychopharmacology* **35**, 169–91 (2010).

Quirk, G. J., Likhtik, E., Pelletier, J. G. & Paré, D. Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. *J Neurosci* **23**, 8800–7 (2003).

Lee, H., Heller, A. S., van Reekum, C. M., Nelson, B. & Davidson, R. J. Amygdala-prefrontal coupling underlies individual differences in emotion regulation. *Neuroimage* **62**, 1875–81 (2012).

Meeuwisse, M. L., Reitsma, J. B., de Vries, G. J., Gersons, B. P. & Ollf, M. Cortisol and post-traumatic stress disorder in adults: systematic review and meta-analysis. *Br J Psychiatry* **191**, 387–92 (2007).

Hakamata, Y. et al. The functional activity and effective connectivity of pulvinar are modulated by individual differences in threat-related attentional bias. *Sci Rep* **6**, 34777 (2016).

Whalen, P. & Phelps, E. *The Human Amygdala*. ( Guilford Press, 2009).

LeDoux, J. E., Iwata, J., Cicchetti, P. & Reis, D. Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear, *J Neurosci* **8**, 2517–29 (1988).

Swanson, L. W. & Petrovic, G. D. What is the amygdala? *Trends Neurosci* **21**, 32–31 (1998).

Shepard, J. D., Barron, K. W. & Myers, D. A. Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. *Brain Res* **861**, 288–95 (2000).

Myers, B. & Greenwood-Van Meerveld, B. Corticosteroid receptor-mediated mechanisms in the amygdala regulate anxiety and colonic sensitivity. *Am J Physiol Gastrointest Liver Physiol* **292**, G1622–9 (2007).

Gainotti, G. Unconscious processing of emotions and the right hemisphere. *Psychoneuroendocrinology* **50**, 205–18 (2012).

Adolphs, R., Jansari, A. & Tranel, D. Hemispheric perception of emotional valence from facial expressions. *Psychophysiology* **15**, 516–24 (2001).

Colnat-Coulbois, S. et al. Tractography of the amygdala and hippocampus: anatomical study and application to selective amygdalectomyparahippocampectomy. *J Neurosurg* **113**, 1135–43 (2010).

Robinson, J. L. et al. Neurofunctional topography of the human hippocampus. *Hum Brain Mapp* **36**, 5018–37 (2015).

Fries, E., Dettenborn, L. & Kirschbaum, C. The cortisol awakening response (CAR): facts and future directions. *Int J Psychophysiol* **72**, 67–73 (2009).

Gaffey, A. E., Bergeman, C. S., Clark, L. A. & Wirth, M. M. Aging and the HPA axis: Stress and resilience in older adults. *Neurosci Biobehav Rev* **68**, 928–45 (2016).

Stalder, T. et al. Assessment of the cortisol awakening response: Expert consensus guidelines. *Psychoneuroendocrinology* **63**, 414–32 (2016).

Okun, M. L. et al. What constitutes too long of a delay? Determining the cortisol awakening response (CAR) using self-report and PSG- assessed wake time. *Psychoneuroendocrinology* **35**, 460–8 (2010).

Oldfield, R. C. The assessment and analysis of handedness: the Edinburgh inventory. *Neuropsychologia* **9**, 97–113 (1971).

Sheehan, D. V. et al. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* **59**, 22–33 (1998).

Derogatis, L. R., Lipman, R. S., Rickels, K., Uhlenhuth, E. H. & Cory, L. The Hopkins Symptom Checklist (HSCL): a self-report symptom inventory. *Behav Med* **19**, 1–7 (1994).

Miller, G. E., Chen, E. & Zhou, E. S. If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. *Psychol Bull* **133**, 25–45 (2007).

Nakano, K. *Stress management*. 2nd ed.; Kongo Press: Tokyo, Japan. (2016).

Nakano, K. & Kitamura, T. The relation of the anger subcomponent of Type A behavior to psychological symptoms in Japanese and foreign students. *Japanese Psychological Research* **43**, 50–54 (2001).

Pruessner, M., Hellehammer, D. H., Pruessner, J. C. & Lupien, S. J. Self-reported depressive symptoms and stress levels in healthy young men: associations with the cortisol response to awakening. *Psychosom Med* **65**, 92–9 (2003).

Vuilleumier, P., Armony, J. L., Driver, J. & Dolan, R. J. Effects of attention and emotion on face processing in the human brain: an event-related fMRI study. *Neuron* **30**, 829–41 (2000).

Tamietto, M. & de Gelder, B. Neural bases of the non-conscious perception of emotional signals. *Nat Rev Neurosci* **11**, 697–709 (2010).

Whitfield-Gabrieli, S. & Nieto-Castanon, A. Conn: a functional connectivity toolbox for correlated and anticorrelated brain networks. *Brain Connect* **2**, 125–41 (2012).

Murphy, K., Birn, R. M., Handwerker, D. A., Jones, T. B. & Bandettini, P. A. The impact of global signal regression on resting state correlation analyses: are anti-correlated networks introduced? *Neuroimage* **44**, 893–905 (2009).

Maldjian, J. A., Laurienti, P. J., Kraft, R. A. & Burdette, J. H. An automated method for neuroanatomic and cytoarchitectonic atlas-based interrogation of fMRI data sets. *Neuroimage* **19**, 1233–9 (2003).

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**Author Contributions**

Y.H. designed the study, wrote the protocol, conducted the psychological assessments and statistical analyses, and wrote the first draft of the manuscript. E.S., S.K., S.M., Y.K., Y.I., and H.T. provided support during the participant recruitment and data collection. S.I. and his research assistants conducted saliva assays. Y.M., Y.M., and T.H. gave technical advice on fMRI analyses. All the authors made critical revisions to the intellectual content of the manuscript and approved the final manuscript.
Additional Information

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