

# Resting-State Functional Connectivity in Patients with Long-Term Remission of Cushing's Disease

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Glucocorticoid disturbance can be a cause of psychiatric symptoms. Cushing's disease represents a unique model for examining the effects of prolonged exposure to high levels of endogenous cortisol on the human brain as well as for examining the relation between these effects and psychiatric symptomatology. This study aimed to investigate resting-state functional connectivity (RSFC) of the limbic network, the default mode network (DMN), and the executive control network in patients with long-term remission of Cushing's disease. RSFC of these three networks of interest was compared between patients in remission of Cushing's disease ( $n=24$ ; 4 male, mean age = 44.96 years) and matched healthy controls ( $n=24$ ; 4 male, mean age = 46.5 years), using probabilistic independent component analysis to extract the networks and a dual regression method to compare both groups. Psychological and cognitive functioning was assessed with validated questionnaires and interviews. In comparison with controls, patients with remission of Cushing's disease showed an increased RSFC between the limbic network and the subgenual subregion of the anterior cingulate cortex (ACC) as well as an increased RSFC of the DMN in the left lateral occipital cortex. However, these findings were not associated with psychiatric symptoms in the patient group. Our data indicate that previous exposure to hypercortisolism is related to persisting changes in brain function.

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## INTRODUCTION

In various stress-related psychiatric disorders such as major depressive disorder and posttraumatic stress disorder (PTSD), alterations in the activity of the hypothalamic–pituitary–adrenal axis (HPA axis) are present, with hyperresponsivity or chronic activation of the HPA axis often resulting in increased levels of cortisol (Carroll *et al*, 1976, 2007; Friedman *et al*, 2007; Inslicht *et al*, 2006; Schlechte *et al*, 1986; Steudte *et al*, 2011; Young and Breslau, 2004). Animals chronically exposed to increased levels of corticosterone display an anxiodepressive-like phenotype, providing a neuroendocrine animal model for stress-related disorders (Darcet *et al*, 2014; David *et al*, 2009). However, extrapolation of findings of this animal model to humans may not always be appropriate, for example when studying human brain activation patterns during specific challenges or resting state.

Cushing's disease represents a unique human model for examining the effects of prolonged exposure to increased levels of endogenous cortisol on brain structure and function, and the relation between these effects and psychiatric symptomatology. In Cushing's disease, hypercortisolism is caused by an adrenocorticotrophic hormone (ACTH) producing pituitary adenoma, stimulating the adrenal glands to continuously release cortisol. In patients with Cushing's disease, a variety of psychiatric symptoms can be induced by hypercortisolism. Major depressive disorder is the most common psychiatric disorder seen in Cushing's disease patients, but comorbidity also includes anxiety, mania, and cognitive dysfunction (Sonino and Fava, 2001). The majority of these symptoms are also seen in stress-related psychiatric disorders, suggesting a common underlying mechanism. After successful biochemical treatment of Cushing's disease, psychiatric symptoms decrease, but patients still show cognitive impairment (Dorn and Cerrone, 2000; Forget *et al*, 2002; Merke *et al*, 2005; Resmini *et al*, 2012; Tiemensma *et al*, 2010b), decreased quality of life (Tiemensma *et al*, 2011), and a higher prevalence of affective disorders and apathy (Tiemensma *et al*, 2010a) compared with healthy controls. These findings suggest that hypercortisolism may cause persisting changes in the brain.

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Effects of hypercortisolism on brain structure include a reduction of hippocampal volume (Bourdeau *et al*, 2002; Simmons *et al*, 2000; Starkman *et al*, 1992) that is reversible after successful abrogation of the hypercortisolism (Bourdeau *et al*, 2002; Starkman *et al*, 1999). In addition, our group found reduced anterior cingulate cortex (ACC) gray matter volumes and increased gray matter volumes in the cerebellum in patients with remission of Cushing's disease (Andela *et al*, 2013), as well as widespread reductions of white matter integrity (van der Werff *et al*, 2014). Remarkably, similar patterns of reduced hippocampal volume (Bremner *et al*, 2003; Campbell *et al*, 2004; Colla *et al*, 2007; Gurvits *et al*, 1996; Kitayama *et al*, 2005; Videbeck and Ravnkilde, 2004), reversibility of hippocampal atrophy after treatment (Boldrini *et al*, 2013; Nordanskog *et al*, 2010; Tendolkar *et al*, 2013; Vermetten *et al*, 2003), and reductions of ACC volumes (Kasai *et al*, 2008; Thomaes *et al*, 2010; Yamasue *et al*, 2003) have been found in stress-related psychiatric disorders.

The effects of hypercortisolism on brain functional connectivity have yet to be investigated. One important network that should be investigated in light of hypercortisolism is the limbic network, consisting of the hypothalamus, hippocampus, amygdala, insula, and parts of the nucleus accumbens (Janes *et al*, 2012; Maclean, 1952). This network is involved in emotional processing and regulation, as well as the encoding of memories. In addition, the components of this network are responsible for the initial stress response and activation of the HPA axis, with subsequent release of corticotropin-releasing hormone (CRH), followed by ACTH that, in turn, stimulates the adrenal glands to secrete cortisol (Shin and Liberzon, 2010). Exposure to severe stress has been related to altered functional connectivity of various components of the limbic network (Admon *et al*, 2009; Brown *et al*, 2014; Jin *et al*, 2014). The network has consistently been found in other resting-state analysis using an ICA approach (Janes *et al*, 2012; Laird *et al*, 2011; Veer *et al*, 2010). However, naming of the network has been less consistent, with studies using the terms limbic network (Janes *et al*, 2012), medial temporal network (Laird *et al*, 2011; Veer *et al*, 2010), and amygdala-hippocampus complex (Veer *et al*, 2010) to indicate the same network. Another network of interest is the default mode network (DMN). This network consists of the precuneus, the posterior cingulate cortex, the medial prefrontal cortex, and parts of the parietal cortex, and is involved in the retrieval and manipulation of episodic memories and semantic knowledge, self-referential processing, and prospective memory (Fox *et al*, 2005; Raichle *et al*, 2001). Alterations in DMN connectivity have been found in stress-related disorders (Bluhm *et al*, 2009a, b; Zhu *et al*, 2012). A third network of interest is the executive control network, consisting of the bilateral dorsolateral prefrontal cortex (PFC), ventrolateral PFC, dorsomedial PFC, and lateral parietal cortices (Beckmann *et al*, 2005; Seeley *et al*, 2007). This network is involved in attention demands, working memory, and cognitive control, and is relevant because of the cognitive impairment in patients with long-term remission of Cushing's disease.

The aim of this study was to examine alterations in resting-state functional connectivity (RSFC) in patients with long-term remitted Cushing's disease. We anticipated to find aberrant RSFC with the limbic network, the DMN, and the

executive control network in these patients compared with matched healthy controls.

## MATERIALS AND METHODS

### Sample Description

All patients with long-term remission of Cushing's disease monitored in a unique cohort at the Leiden University Medical Center ( $n = 49$ ) and between 18 and 60 years of age were invited by letter and those who did not respond were contacted by phone. The response rate was 96%. Thirty-one patients were willing to participate and were screened for eligibility. Six patients were excluded because of one of the following exclusion criteria: a (history of) drug or alcohol abuse, neurological problems, contraindications for undergoing a magnetic resonance imaging (MRI) scan and left-handedness. Healthy controls were pair-wise matched to the patients based on gender, age, and education and recruited by advertisements in grocery stores and via Internet. In addition to the general exclusion criteria of the study, a history or presence of a psychiatric disorder was an exclusion criterion for the control group. At the day of the MRI scan, patients were asked again about a lifetime history of psychiatric disorders. One patient reported a history of major depressive disorder before the suspected onset of the Cushing's disease.

A total of 25 patients with remission of Cushing's disease and 25 matched healthy controls were included in this study. The MRI scanning session of one patient was aborted prematurely before the resting-state scan was acquired. Therefore, we excluded data from this patient and the matched healthy control from the current study, resulting in a total sample size of 24 patients and 24 controls. The diagnosis of active Cushing's disease had been confirmed in all patients, following previously described criteria (Tiemensma *et al*, 2010b). All patients were treated with transsphenoidal surgery. Thirteen patients remained glucocorticoid dependent after surgery and were substituted with hydrocortisone (on average 20 mg/day, divided into three dosages). Remission of the Cushing's disease was confirmed in the 11 other patients. The estimated duration of disease was determined through patients' history by looking for the earliest physical/somatic signs. Duration of remission was calculated from the date of curative transsphenoidal surgery, or in case of persistent disease, from the date of normalization of biochemical tests after postoperative radiotherapy. Demographics and patient characteristics are reported in Table 1. Written informed consent was obtained from all participants before the clinical assessment and the MRI scan session. The medical ethical committee of the Leiden University Medical Center approved the study protocol.

### Clinical Data Acquisition

Presence and severity of depressive symptoms were evaluated using the Montgomery-Åsberg depression rating scale (MADRS) (Montgomery and Åsberg, 1979) and the Inventory of Depression Symptomatology (IDS) (Rush *et al*, 1996). Anxiety was evaluated using the Beck Anxiety Inventory (BAI) (Beck *et al*, 1988) and the Fear Questionnaire (FQ) (Marks and Mathews, 1979). Apathy and irritability were assessed using the Apathy Scale (AS) (Starkstein *et al*, 2001)

**Table 1** Demographics of the Total Sample and Clinical Characteristics of the Patients with Long-Term Remission of Cushing's Disease

Characteristics	Patients with long-term remission of Cushing's disease (n = 24)	Matched healthy controls (n = 24)
Gender (male/female)	4/20	4/20
Age (years), mean (SD)	44.96 (7.52)	46.5 (7.06)
<i>Education, n (%)</i>		
Low	6 (25%)	6 (25%)
Intermediate	11 (45.8%)	10 (41.7%)
High	7 (29.2)	8 (33.3%)
<i>Surgery, n (%)</i>		
Transsphenoidal adenomectomy	24 (100%)	
Bilateral adrenalectomy	2 (8.3%)	
Radiotherapy, n (%)	6 (25%)	
Disease duration (years), mean (SD)	8.30 (8.12)	
Duration of remission (years), mean (SD)	10.92 (8.36)	
<i>Hypopituitarism, n (%)</i>		
Any axis	14 (58.3%)	
GH	10 (41.7%)	
LH/FSH	9 (37.5%)	
TSH	10 (41.7%)	
ADH	3 (12.5%)	
Hydrocortisone substitution	13 (54.2%)	
<i>Clinical severity index (CSI), mean (SD)</i>		
Active phase, total	8.08 (2.02)	
Remission phase, total	2.50 (1.53)	

and the Irritability Scale (IS) (Chatterjee *et al*, 2005), respectively. The Cognitive Failures Questionnaire (CFQ) (Broadbent *et al*, 1982) was used to assess cognitive functioning. The Cushing's syndrome Severity Index (CSI) (Sonino *et al*, 2000) was used to assess current severity of symptoms and to retrospectively estimate (clinical) severity at the time of active disease.

The MADRS was assessed before scanning on the scanning day. For all other questionnaires, the time between the scan and filling out the questionnaires was on average  $1.4 \pm 3.2$  days (range 0–19). This broad range was mainly caused by one outlier of 19 days, whereas all other participants filled out questionnaires within 1 week. Moreover, 83% of the participants filled out questionnaires within 1 day after scanning.

### MRI Data Acquisition

Imaging data were acquired on a Philips 3T magnetic resonance imaging system (software version 3.2.1; Philips Healthcare, Best, The Netherlands). A SENSE-32 channel

head coil was used for radio frequency transmission and reception. Beforehand, the participants were instructed to lie as still as possible, with their eyes closed and without falling asleep. After completion of the scan, all participants confirmed not having fallen asleep. Resting-state fMRI (RS-fMRI) data were acquired using T2\*-weighted gradient-echo echo-planar imaging with the following scan parameters: 200 whole-brain volumes, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms, flip angle = 80°, 38 axial slices, matrix size = 80 × 80, voxel size = 2.75 × 2.75 × 2.75 mm, slice gap = 0.275, scan duration = 449 s.

A high-resolution anatomical image (T1-weighted sequence; TR = 9.8 ms, TE = 4.6 ms, 140 axial slices, matrix size = 256 × 256, voxel size 1.17 × 1.17 × 1.2 mm, no slice gap, scan duration = 296 s), and a high-resolution T2\*-weighted gradient echo EPI scan (TR = 2200 ms, TE = 30 ms, flip angle = 80°, 84 axial slices, matrix size = 112 × 112, voxel size = 1.96 × 1.96 × 2 mm, no slice gap, scan duration = 46.2 s) were acquired for registration to standard space.

A neuroradiologist, blinded to the status of the subjects, examined all anatomical images. Apart from incidental age-related white matter hyperintensities and minor effects of the posttranssphenoidal surgery in the perisellar area, no other macroscopic abnormalities were observed in the patients and controls.

### Data Preprocessing

All MRI data were processed using FSL (FMRIB's Software Library, [www.fmrib.ox.ac.uk/fsl/](http://www.fmrib.ox.ac.uk/fsl/); version 5.0.6; Smith *et al*, 2004). Non-brain tissue removal was applied to the high-resolution T1-weighted image and the high-resolution EPI image. The preprocessing of the RS-fMRI was carried out using FMRI Expert Analysis Tool (FEAT). Motion correction was applied to the RS-fMRI data along with non-brain tissue removal, spatial smoothing using a 6-mm full-width at half-maximum (FWHM) Gaussian kernel, grand-mean intensity normalization of the entire 4D data set by a single multiplicative factor and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, 0.01 Hz cutoff).

The RS-fMRI data of each participant were then registered to their respective high-resolution EPI images. The high-resolution EPI image was registered to the T1-weighted image, and the T1-weighted image to MNI-152 standard space image (T1-weighted standard brain averaged over 152 subjects; Montreal Neurological Institute, Montreal, QC, Canada), with a resampled resolution of 4 mm.

### Extracting Resting-State Networks

Analysis was carried out using Probabilistic Independent Component Analysis (PICA; Beckmann and Smith, 2004) as implemented in MELODIC (Multivariate Exploratory Linear Decomposition into Independent Components). We opted to use this method over a seed-based approach, because our hypotheses were aimed at investigating connectivity with networks rather than with more localized areas. In addition, PICA gives us the advantage to distinguish physiological signals from noise, as they are separated in different components. This technique also enables us to omit global signal regression that is known to induce negative connectivity between networks (Murphy *et al*, 2009). Default group PICA

processing steps were applied to the individual preprocessed and normalized data sets: masking of non-brain voxels, voxel-wise de-meaning of the data, and normalization of the voxel-wise variance based on all data sets. Subsequently, the preprocessed data were concatenated in time to create a single 4D data set that was then projected into a 20-dimensional subspace using principal component analysis. The observations were decomposed into 20 sets of independent vectors that describe signal variation across the temporal (time courses) and spatial (maps) domains by optimizing for non-Gaussian spatial source distributions using a fixed-point iteration technique (Hyvarinen, 1999). We chose to use 20 independent components to reach the same balance between the amount of clustering and splitting as previous studies applying the same techniques (Smith *et al*, 2009a). The resulting estimated component maps were divided by the SD of the residual noise and thresholded at a probability threshold of  $p > 0.5$  (ie, an equal weight is placed on false positives and false negatives) by fitting a mixture model to the histogram of intensity values.

### Statistical Analyses

The set of spatial maps from the group-average analysis was used to generate subject-specific versions of the spatial maps, and associated time courses, using dual regression (Filippini *et al*, 2009). In short, for each subject, the group-average set of spatial maps was regressed as spatial regressors in a multiple regression into the preprocessed individual 4D resampled data set. This resulted in a subject-specific time course for each component separately. Next, those time courses were regressed as temporal regressors in a multiple regression into the same preprocessed individual 4D data set, resulting in a set of subject-specific spatial maps, one for each of the 20 components. In addition, subject-specific  $z$ -maps for each of the components were constructed through normalization of the subject-specific spatial maps by the residual within-subject noise.

Upon visual inspection, we selected three components based on spatial similarity to functional networks described before: the limbic network (Shin and Liberzon, 2010), the DMN (Beckmann *et al*, 2005; Fox *et al*, 2005; Raichle *et al*, 2001), and the executive control network (Seeley *et al*, 2007). We segmented the 1 mm MNI-152 standard brain into gray matter, white matter, and cerebrospinal fluid (CSF) and created a mask comprising gray and white matter only. This mask was then registered to an isotropic resolution of 4 mm and applied to the study-specific mask in order to exclude voxels containing CSF from our analyses.

The subject-specific spatial maps of each of these three components along with the study-specific gray and white matter mask were fed into FSL's Randomise tool. The groups were compared using a general linear model (GLM) including age and level of education as confound regressors. In addition, for each subject, gray matter density maps were derived from the anatomical scans using FSL. Previously, Cushing's disease-related gray matter abnormalities were reported in the same sample (Andela *et al*, 2013). Therefore, to control for structural abnormalities possibly confounding differences in functional connectivity and to correct for the effects of possible misregistration (Oakes *et al*, 2007), information about gray matter values of each subject was

included as a voxel-wise confound regressor in the GLM. Between- and within-group effects were tested using permutation-based (5000 permutations) nonparametric testing. To control for family-wise error we applied threshold-free cluster enhancement (TFCE; (Smith and Nichols, 2009b), and statistical maps were thresholded at  $p < 0.05$ .

## RESULTS

### Psychopathology Scores

The psychometric data are reported in Table 2. To correct for multiple comparisons we applied a Bonferroni correction and adjusted the level of significance to  $p < 0.005$ . Comparisons of the psychometric data between patients with long-term remission of Cushing's disease and the matched healthy controls showed significant differences on the MADRS ( $p < 0.001$ ) and AS ( $p = 0.001$ ). The following psychometric data showed uncorrected differences between groups: IDS ( $p = 0.008$ ), BAI ( $p = 0.010$ ), CFQ ( $p = 0.028$ ), and FQ ( $p = 0.006$ ). The only subscale of the FQ that showed an uncorrected difference between groups was the social phobia subscale ( $p = 0.012$ ).

### Resting-State Functional Connectivity

Motion parameters (Table 2) did not differ significantly between groups for both absolute displacement ( $t = -1.017$ ,  $p = 0.315$ ) and relative displacement ( $t = 0.355$ ,  $p = 0.725$ ). The three networks of interest were identified among the 20 components resulting from the PICA (Figure 1). These three networks have been consistently present across subjects and over time using PICA in previous studies (Beckmann *et al*, 2005; Veer *et al*, 2010). All three functional networks were present in both the patient group and healthy control group. Between-group differences in RSFC for each network were examined by comparing the following contrasts: (1) controls <patients and (2) controls >patients. These comparisons revealed a between-group difference in RSFC between the limbic network and the subgenual subregion of the ACC ( $p < 0.05$ , TFCE corrected; Figure 2a). To determine whether this effect was driven by a decrease in negative connectivity or an increase in positive connectivity, mean individual  $z$ -scores for this region were extracted from the subject-specific  $z$ -maps of the limbic network component. Comparison of the mean  $z$ -scores of both groups showed an increase in positive connectivity of the limbic network with the subgenual ACC in patients with Cushing's disease in remission compared with healthy controls (Figure 2b). To determine whether the strength of RSFC between the limbic network and the subgenual ACC was associated with psychopathology scores of the patients with long-term remission of Cushing's disease, we performed linear regression analyses with adjustment for age and education. The psychopathology scores reported in Table 2, as well as the active and the remission subscales of the CSI, and the disease and remission duration in years did not show a significant association with the strength of the RSFC between the limbic network and the subgenual ACC in patients with long-term remission of Cushing's disease (for all:  $p > 0.1$ ). Furthermore, the found effect could not be related to treatment with hydrocortisone as there was no significant difference in  $z$ -scores between the

**Table 2** Symptom Severity Scores of Patients with Long-Term Remission of Cushing's Disease Versus Matched Healthy Controls

	Patients with long-term remission of Cushing's disease (n = 24)	Matched healthy controls (n = 24)	P-value
Montgomery-Åsberg Depression Rating Scale (MADRS), mean (SD)	6.37 (5.59)	1.42 (1.79)	<0.001 <sup>a,**</sup>
Inventory of Depression Symptomatology (IDS), mean (SD)	47.00 (13.23)	36.29 (5.85)	0.008 <sup>a,**</sup>
Beck Anxiety Inventory (BAI), mean (SD)	28.48 (5.79)	24.33 (3.16)	0.010 <sup>a,**</sup>
Fear Questionnaire (FQ), mean (SD)	25.52 (17.00)	13.92 (9.72)	0.006 <sup>b,**</sup>
Agoraphobia subscale, mean (SD)	6.35 (7.98)	2.67 (3.33)	0.209 <sup>a</sup>
Blood injury phobia subscale, mean (SD)	6.48 (8.40)	3.46 (4.13)	0.270 <sup>a</sup>
Social phobia subscale, mean (SD)	12.70 (7.77)	7.79 (4.82)	0.012 <sup>b,**</sup>
Irritability scale (IS), mean (SD)	12.13 (8.91)	8.54 (6.02)	0.176 <sup>a</sup>
Apathy scale (AS), mean (SD)	13.91 (6.50)	8.13 (3.80)	0.001 <sup>b,**</sup>
Cognitive failure questionnaire, mean (SD)	37.39 (16.55)	28.58 (9.10)	0.028 <sup>b,**</sup>
Motion parameters in mm			
Absolute Displacement in mm, mean (SD)	0.29 (0.14)	0.35 (0.22)	0.315 <sup>b</sup>
Relative Displacement in mm, mean (SD)	0.12 (0.06)	0.12 (0.05)	0.725 <sup>b</sup>

\* $P < 0.05$ , uncorrected; \*\* $P < 0.005$ , Bonferroni corrected.

<sup>a</sup>Mann-Whitney *U*-test.

<sup>b</sup>Independent sample *t*-test.

13 patients who were treated and those who were not ( $t = 0.396$ ;  $p = 0.696$ ).

Analyses of between-group differences in RSFC with the DMN revealed aberrant connectivity with a small cluster located in the superior division of the left lateral occipital cortex ( $p < 0.05$ , TFCE corrected; Figure 3a). Examination of the individual *z*-scores showed positive connectivity of this area with the DMN in the patients and negative connectivity in the healthy control group (Figure 3b). Linear regression analyses with adjustment for age and education did not show a significant association between individual *z*-scores and the symptom scores of the behavioral measurements as well as the subscales of the CSI and the disease and remission duration in the patients with long-term remission of Cushing's disease. In addition, there was no difference in *z*-scores between patients with and without hydrocortisone treatment ( $t = 0.063$ ;  $p = 0.950$ ). Contrasts testing the between-group differences in RSFC of the executive control network did not show any effects.

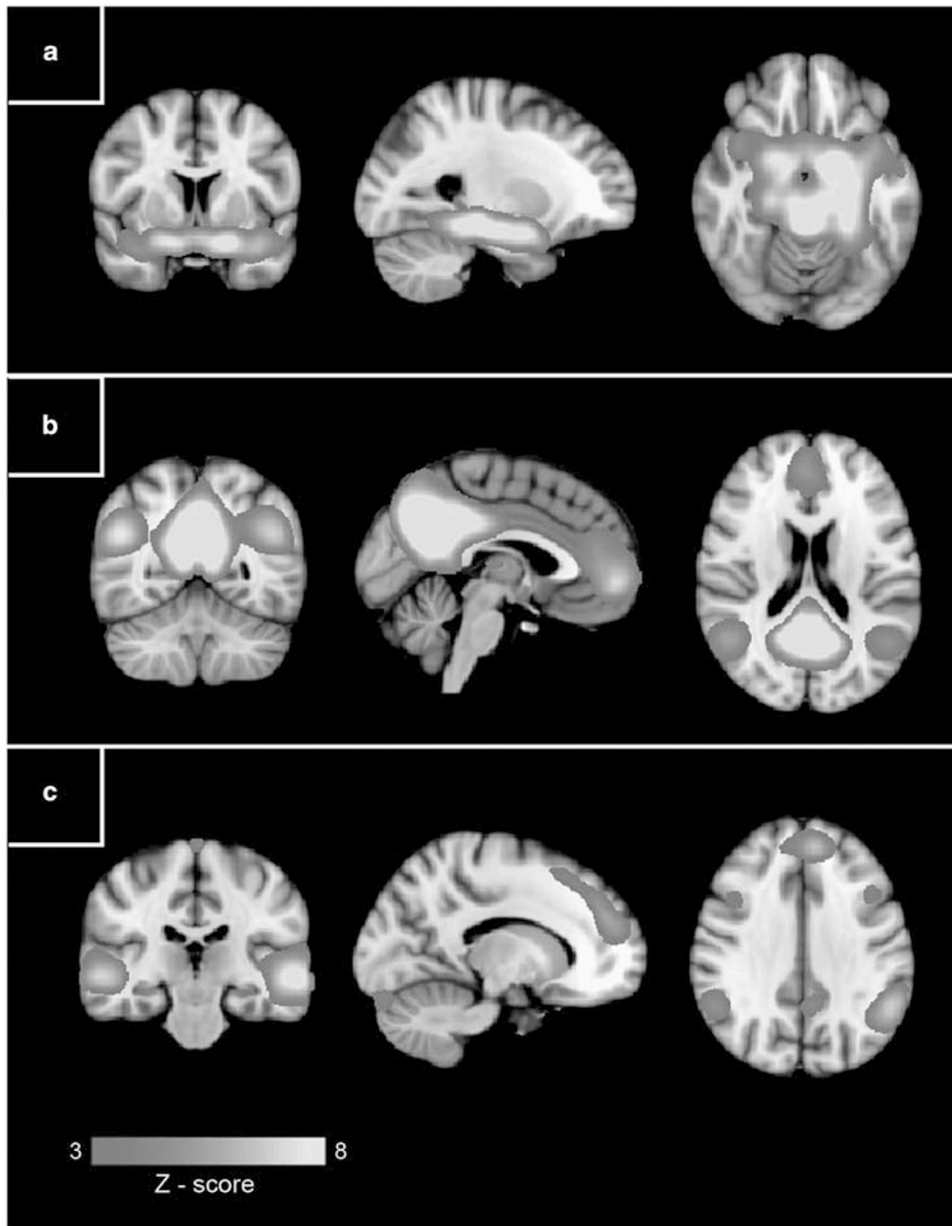
Omitting the voxel-wise gray matter covariate from the statistical model did not change the functional connectivity results as described in the previous section. This indicates that the altered RSFC within the three networks is unlikely to be related to macroscopic (ie, MRI observable) gray matter abnormalities.

## DISCUSSION

We examined RSFC in patients with long-term remission of Cushing's disease and hypothesized to find aberrant RSFC of three networks of interest including the limbic network, the DMN, and the executive control network in patients with long-term remission of Cushing's disease compared with matched healthy controls. Our hypothesis was confirmed for both the limbic network and the DMN, but not for the executive control network.

In patients we found an increase in RSFC between the limbic network and the subgenual ACC and an increase in RSFC between the DMN and a small cluster located in the superior division of the left lateral occipital cortex. Animal studies suggest that the subgenual ACC is a target site for the negative feedback effects of glucocorticoids on stress-induced HPA axis activity. Damage to this location leads to a diminished ability to regulate and inhibit HPA axis activity (Diorio *et al*, 1993). This has also been observed in humans, in whom behavioral studies showed that the subgenual ACC function is involved in emotion regulation (Bush *et al*, 2000; Phillips *et al*, 2003a; Quirk and Beer, 2006; Shin *et al*, 2000). The subgenual ACC exerts top-down inhibitory control over subcortical structures, most importantly the amygdala (Kim *et al*, 2011). Failing to exert this inhibitory control could lead to the disturbed emotion regulation seen in stress-related psychiatric disorders (Johnstone *et al*, 2007; Patel *et al*, 2012; Phillips *et al*, 2003b). Therefore, the subgenual ACC is the target site for some specific forms of treatment for stress-related disorders. Deep brain stimulation of the subgenual ACC is currently used as a last resort treatment for treatment-resistant depression (Johansen-Berg *et al*, 2008; Lozano *et al*, 2008; Mayberg *et al*, 2005). Furthermore, subgenual ACC activity has been found to change under influence of fluoxetine use (Mayberg *et al*, 2000), an antidepressant that is used as a treatment for both PTSD and depression. These studies suggest that targeting subgenual ACC function is an effective treatment element for stress-related psychiatric disorders.

Our finding of increased RSFC connectivity between the limbic network and the subgenual ACC in patients with long-term remission of Cushing's disease suggest that these changes in coupling are established under the influence of hypercortisolism. This hypothesis is further supported by a study by Veer *et al* (2012) who found that RSFC connectivity between the amygdala (a key component of the limbic network) and the subgenual ACC is reactive to endogenous



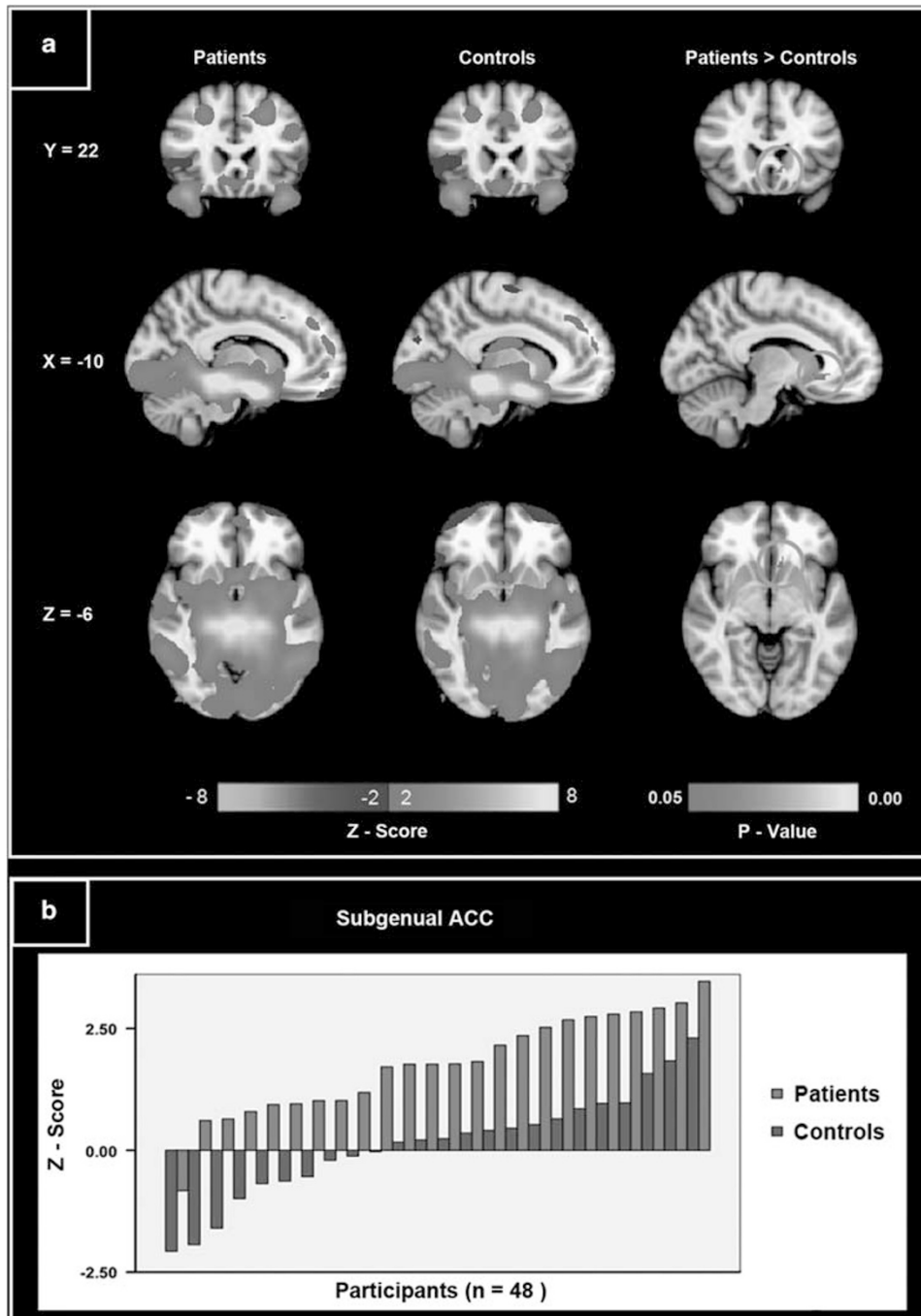
**Figure 1** Resting-state networks of interest. (a) The limbic network. (b) The default mode network. (c) The executive control network. Images are z-statistics, overlaid on the MN1-152 1 mm standard brain. The left hemisphere of the brain corresponds to the right side of the coronal and transversal images.

cortisol. In addition, our findings indicate that elevated RSFC between the subgenual ACC and key regions of the limbic network (like amygdala and insula) in depression (Connolly *et al*, 2013) and PTSD (Brown *et al*, 2014; Gilboa *et al*, 2004) could be an effect of exposure to high levels of cortisol often accompanying these stress-related disorders.

It is well known that optimal HPA axis reactivity is crucial to make correct adaptive responses in challenging situations but, once out of balance, can also be related to vulnerability or the emergence of psychiatric symptoms. We did not find an association of any of the behavioral measurements with the strength of the connectivity between the limbic network and the subgenual ACC. As our study was designed to test group differences, our study could have been underpowered to test for this type of associations. Therefore, it remains

unknown whether these increases in connectivity reflect or underlie psychiatric symptoms, or more adaptive behavior. Recently, the latter has been suggested for increased RSFC between the hippocampus and the ventromedial prefrontal cortex in a group of trauma-exposed combat paramedics (Admon *et al*, 2013).

We also found an increase in RSFC in the patient group between the DMN and a small cluster located in the superior division of the left lateral occipital cortex. This area is situated adjacent to parietal regions that are part of the DMN. Therefore, an interpretation could be that the DMN of the patients recruits larger areas compared with healthy controls. This may indicate a less efficient use of the DMN in patients with long-term remission of Cushing's disease.

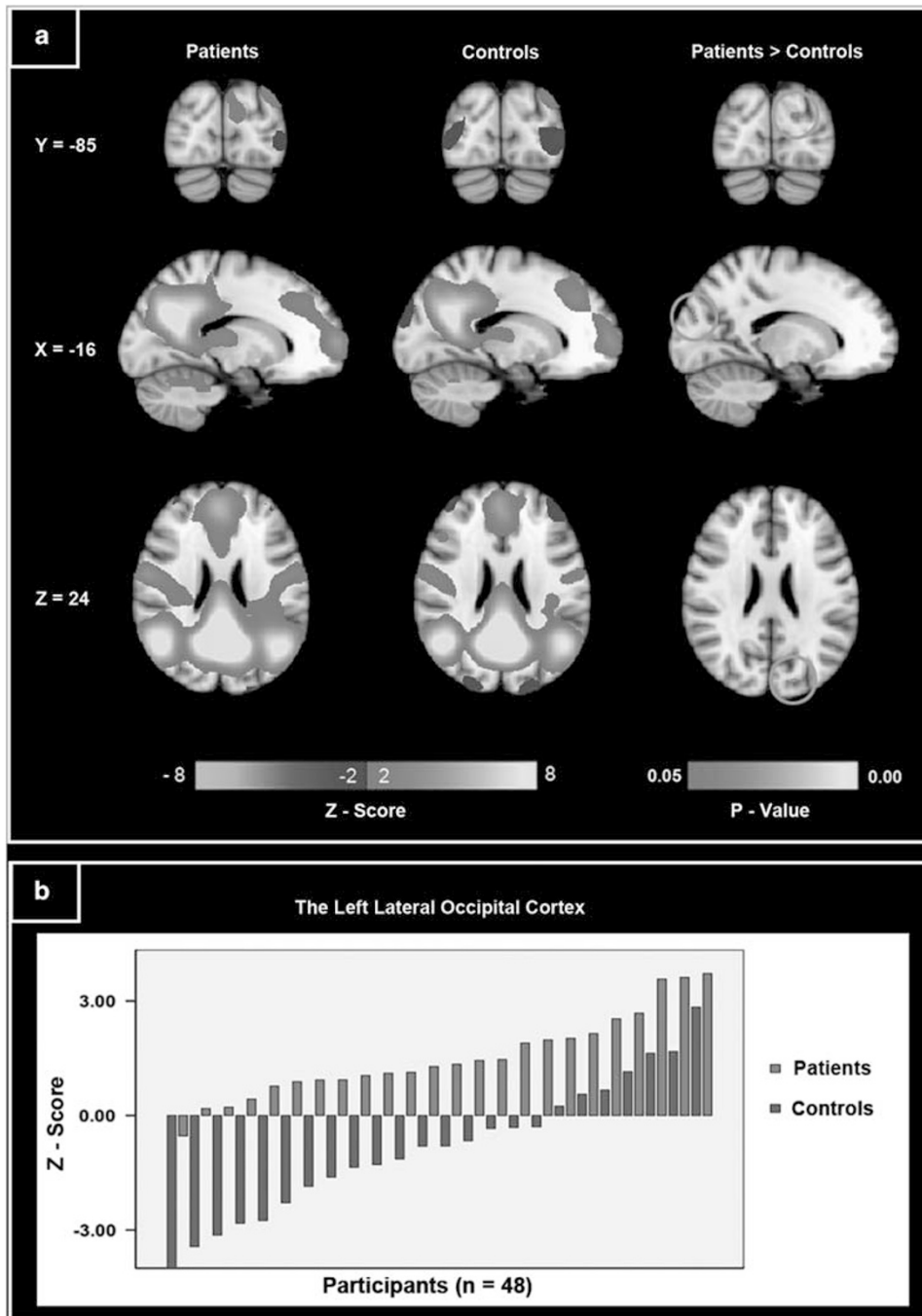


**Figure 2** Resting-state functional connectivity of the limbic network. (a) Depicted here are the within-group mean z-scores and the between-group effect for the limbic network. Images representing the statistics are overlaid on the MNI-152 1 mm standard brain. The between-group effect is displayed as  $p$ -statistics, TFCE corrected for family-wise error ( $p < 0.05$ ). The between-group effect shows increased RSFC between the limbic network and the subgenual ACC. (b) Distribution of the mean individual z-scores within the subgenual ACC. Depicted in red are the patients with long-term remission of Cushing's disease, depicted in blue are the matched healthy controls, and both are sorted from smallest to highest z-score. A full colour version of this figure is available at the *Neuropsychopharmacology* journal online.

Contrary to our expectations, the executive control network did not show differences in RSFC between the patients and controls. However, the observed cognitive impairment still present in the patients with Cushing's disease in remission was subtle, and the cognitive demands during resting state are low because of the absence of specific goal-oriented tasks. Consequently, differences in functional activity might only be expressed when demands on cognition

are high (ie, during tasks). Task-related fMRI studies with a high cognitive load could be used to investigate this, as these tasks also show aberrant activity patterns in the executive control network in patients with depression and PTSD (Aizenstein *et al*, 2009; Daniels *et al*, 2010; Wang *et al*, 2008).

Limitations of our study consist of the cross-sectional design of our study that does not permit any causal



**Figure 3** Resting-state functional connectivity of the default mode network. (a) Depicted here are the within-group mean z-scores and between-group effect for the default mode network. Images representing the statistics are overlaid on the MNI-152 1 mm standard brain. The between-group effect is displayed as *p*-statistics, TFCE corrected for family-wise error ( $p < 0.05$ ). The between-group effect shows increased RSFC between the default mode network and a small cluster located in the superior division of the left lateral occipital cortex. (b) Distribution of the mean individual z-scores within the superior division of the left lateral occipital cortex. Depicted in red are the patients with long-term remission of Cushing's disease, depicted in blue are the matched healthy controls, and both are sorted from smallest to highest z-score.

conclusions to be drawn from the data. Therefore, we cannot rule out that the found between-group differences preceded the onset of the Cushing's disease. It is also possible that the difference in connectivity is an effect of the depressive symptomatology in the patient group that developed independent of the exposure to hypercortisolism. However, we did not find a correlation between depression severity and the strength of connectivity, making it less likely that the

difference in connectivity purely reflects psychopathology. Another limitation is that we did not correct for the number of networks investigated in this study, thereby giving the study an explorative nature. Finally, we have not applied global signal regression that is known to induce negative connectivity between networks (Murphy *et al*, 2009). However, this also means our method is less sensitive toward detecting negative connectivity in the data.



In summary, findings from neuroendocrine animal models for stress-related disorders using rodents with chronically elevated levels of corticosterone may have limited applicability to human brain function during challenges or resting state. Cushing's disease represents a unique model for investigating effects of prolonged hypercortisolism on the human brain. Earlier studies using Cushing's disease to investigate the long-term effects of hypercortisolism on the structure of the brain showed smaller gray matter volumes of the ACC, increased gray matter volumes of the cerebellum, and reduced structural connectivity to be rather persistent (Andela *et al*, 2013; van der Werff *et al*, 2014), whereas normalization of cortisol levels reversed hippocampal atrophy following treatment of Cushing's disease (Starkman *et al*, 1999). Our study is the first to suggest that exposure to hypercortisolism may lead to persistent changes in brain functional connectivity as well. Our finding of increased RSFC between the limbic network and the subgenual ACC is similar to the results of studies performed in patients with stress-related disorders, suggesting that these changes in RSFC in these stress-related disorders are established under the influence of increased levels of cortisol.

Future studies in the effects of stress or cortisol on RSFC should focus on connectivity between the limbic network (or specific key regions of this network) and the subgenual ACC in order to replicate this finding. This could also be achieved by conducting a targeted approach using the subgenual ACC as seed to investigate connectivity differences. Longitudinally designed studies should be performed to investigate the causal effects of hypercortisolism on RSFC. In addition, task-related fMRI paradigms could contribute to investigate changes in brain activity during specific demands.

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