Understanding clinical outcome in patients with pituitary disease: a biopsychosocial approach

Andela, C.D.; Andela C.D.

CHAPTER 4

Altered neural processing of emotional faces in remitted Cushing's disease

Janna Marie Bas-Hoogendam*, Cornelie D. Andela* , Steven J.A. van der Werff, J. Nienke Pannekoek, Henk van Steenbergen, Onno C. Meijer, Mark A. van Buchem, Serge A.R.B. Rombouts, Roos C. van der Mast, Nienke R. Biermasz, Nic J.A. van der Wee, and Alberto M. Pereira

** Equally contributed*

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ABSTRACT

Patients with long-term remission of Cushing's disease (CD) demonstrate residual psychological complaints. At present, it is not known how previous exposure to hypercortisolism affects psychological functioning in the long-term. Earlier Magnetic Resonance Imaging (MRI) studies demonstrated abnormalities of brain structure and resting-state connectivity in patients with long-term remission of CD, but no data are available on functional alterations in the brain during the performance of emotional or cognitive tasks in these patients.

We performed a cross-sectional functional MRI study, investigating brain activation during emotion processing in patients with long-term remission of CD. Processing of emotional faces versus a non-emotional control condition was examined in 21 patients and 21 matched healthy controls. Analyses focused on activation and connectivity of two a priori determined regions of interest: the amygdala and the medial prefrontal - orbitofrontal cortex (mPFC-OFC). We also assessed psychological functioning, cognitive failure, and clinical disease severity.

Patients showed less mPFC activation during processing of emotional faces compared to controls, whereas no differences were found in amygdala activation. An exploratory psychophysiological interaction analysis demonstrated decreased functional coupling between the ventromedial PFC and posterior cingulate cortex (a region structurally connected to the PFC) in CD-patients.

The present study is the first to show alterations in brain function and task-related functional coupling in patients with long-term remission of CD relative to matched healthy controls. These alterations may, together with abnormalities in brain structure, be related to the persisting psychological morbidity in patients with CD after long-term remission.

INTRODUCTION

Cushing's disease (CD) is characterized by elevated endogenous cortisol levels and is related to physical and psychological morbidity in more than 70% of the patients (1). After correction of hypercortisolism, physical and psychological symptoms improve substantially. However, patients with long-term remission of CD still demonstrate residual physical and psychopathological morbidity (2,3), impairments in cognitive functioning (4–7) and reduced quality of life (8). A recent study provided evidence for a role of specific genetic polymorphisms in the etiology of cognitive impairments in these patients (9), but the persistent symptoms in patients with long-term remission of CD are still ill-understood. Cortisol acts in the central nervous system by stimulation of mineralocorticoid receptors and glucocorticoid receptors. An appropriate balance in activation of these two receptor systems is required for adequate stress responses. Hyperactivation of the hypothalamic-pituitary-adrenal (HPA)-axis during active CD not only induces overactivation of the receptors, but also an imbalance in mineralocorticoid- and glucocorticoid receptor activation, both of which might result in inadequate stress responses and enhanced vulnerability to psychopathology (10). The residual psychological and cognitive morbidity after long-term remission of CD suggests that exposure to hypercortisolism not only has acute effects, but might also be related to persistent changes in the brain.

Several neuroimaging studies have observed changes in morphology and function of the brain during the active phase of CD (11). Using functional Magnetic Resonance Imaging (fMRI), less activation in the left anterior superior temporal gyrus and higher activation in frontal, medial, and subcortical regions during the identification of emotional faces was measured, indicating altered activity of brain structures relevant to the perception, processing and regulation of emotion (12). In addition, adolescents with active CD demonstrated increased activation in the left amygdala and right anterior hippocampus during a memory task involving emotional faces (13). Moreover, patients with active CD showed structural brain abnormalities, including hippocampal volume reduction and cerebral atrophy (14,15). Mainly short term follow-up studies (duration of follow-up: 6-40 months) demonstrated at least partly reversibility of these structural brain abnormalities (14,16), although no firm conclusions can be drawn about the completeness of reversibility since long-term follow-up studies are lacking. Recently, we and others have shown that patients with long-term remission of CD (mean duration of remission: 11.2 years) still have abnormalities in brain structure, as evidenced by smaller grey matter volumes in the anterior cingulate cortex, larger grey matter volumes in the left lobe of the cerebellum (17) and widespread reductions in white matter integrity (18). In addition, these patients showed increased resting-state functional connectivity of the anterior cingulate cortex (19). Furthermore, a spectroscopy study by Resmini and colleagues demonstrated persistent biochemical alterations in both the left and right hippocampus in cured CD patients (20). Taken together, these findings indicate that patients with long-term remission of CD have persisting structural and biochemical brain

abnormalities, as well as changes in functional connectivity at rest, after cure of previous hypercortisolism (11). However, it is presently unknown whether these alterations appear in conjunction with altered brain activity patterns during the performance of cognitive or emotional tasks.

Given the link between hypercortisolism and disturbances in the stress response (10), and the irritability, anxiety, and depressive symptoms reported by patients with long-term remission of CD (2), we decided to examine brain activity during the processing of emotional faces in these patients. Patients were part of the sample described previously (17–19). Focus was on two regions of interest (ROIs): the amygdala and the medial prefrontal – orbitofrontal cortex (mPFC-OFC) (21). The amygdala and the mPFC, including the orbitofrontal cortex, are both part of the limbic system and involved in the regulation of the HPA-axis (22). Previous neuroimaging studies in patients with stress-related psychiatric disorders demonstrated hyperactivation of the amygdala and hypoactivation of the mPFC in response to emotional stimuli (23,24), and it has been suggested that disturbances in the amygdala – mPFC circuitry lead to symptoms of anxiety (22). Considering the similarity in psychopathology between patients with CD and patients suffering from stress-related psychiatric disorders, we hypothesized that patients with long-term remission of CD would also show hypoactivaton of the mPFC combined with hyperactivation of the amygdala, relative to matched controls.

In addition to the ROI analyses, we performed a whole-brain analysis to examine task-related activation in other brain regions. Furthermore, we investigated potential associations between brain activity and psychological and cognitive measures, and several clinical characteristics (e.g. hydrocortisone dependency and disease severity). In addition, we used psychophysiological interaction analyses (25) to explore group differences in functional connectivity during processing of emotional faces.

MATERIAL AND METHODS

Participants

Patients with long-term remission of CD of pituitary origin, monitored yearly at our institute, were invited by letter to participate in this study (n=49; age 18-60 years). Patients who did not respond to the invitation letter were contacted by phone. Thirty-one CD-patients were willing to participate and were screened for eligibility. Exclusion criteria were past or present drug- or alcohol abuse, neurological disorders, general contraindications for undergoing a magnetic resonance imaging (MRI) scan and left-handedness. Healthy control participants were recruited by advertisements in grocery stores and via Internet and were included based on the following inclusion criteria: no neurological or psychiatric disorders (past or present), no psychotropic medication, right-handedness and no contraindications for MRI-scanning. A total of 25 CD-patients and 30 controls took part in this study. Three CD-patients and

eight controls were excluded from the final analyses because of behavioral data indicating insufficient task participation (see Analysis behavioral data). Next, the remaining CD-patients and controls were matched on gender, age and education, resulting in a final sample of 21 CD-patients and 21 controls.

The diagnosis of CD had been confirmed in all patients. Criteria for diagnosis as well as for biochemical cure were applied as previously described (4). Duration of disease was estimated by looking for the earliest physical/somatic signs in the patient's history. The 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. Written informed consent was obtained from all participants prior to the clinical assessment and the MRI-scan. The study protocol was approved by the medical ethical committee of the Leiden University Medical Center.

Study procedure

Each participant visited the Leiden University Medical Center for a two-hour session consisting of an interview for the evaluation of clinical data, assessment of psychopathology and cognitive functioning, and an MRI-scan. At the end of the session, participants were asked to complete several self-rating questionnaires at home for the assessment of psychopathology and cognitive functioning and to return them within a week. One CD-patient did not return the questionnaires.

Assessment of psychopathology and cognitive functioning

The assessment of psychopathology and cognitive functioning took place as described earlier (17,18). Presence and severity of depressive symptoms was assessed using the Montgomery-Åsberg Depression Rating Scale (MADRS; (26)) and the Inventory of Depression Symptomatology (IDS; (27)). Anxiety was evaluated using the Beck Anxiety Inventory (BAI; (28)) and the Fear Questionnaire (FQ; (29)). Apathy and irritability were assessed using the Apathy Scale (AS; (30)) and the Irritability Scale (IS; (31)). Failures in perception, memory, and motor function were evaluated using the Cognitive Failures Questionnaire (CFQ; (32)).

Cushing's syndrome Severity Index (CSI)

The Cushing's syndrome Severity Index (CSI; (33)) was used to evaluate current severity of symptoms and to retrospectively estimate clinical severity at the time of active disease. A higher total score on the CSI indicates greater disease severity. The information necessary for completing this index was derived from clinical history and medical files. Two raters, who reached consensus on each feature in case of discrepancy, scored the CSI.

The Faces task

The fMRI Faces task was based on the event-related emotional faces paradigm reported by (34); the task described here has been employed earlier (35,36). The task presents 120 color photographs of faces with angry, fearful, happy, neutral and sad expressions (task-condition) and 80 scrambled faces (control-condition). Photographs were selected from the Karolinska Directed Emotional Faces System (37) and represented standardized facial expressions of emotions expressed by amateur actors. For each facial expression, 24 photographs were selected (12 male faces, 12 female faces). Duration of stimulus presentation (both of faces and scrambled faces) was 2.5 s. Between stimuli, a black screen was presented with a random duration between 0.5 and 1.5 s. An event-related design was used to reduce anticipatory effects. During the task-condition, participants were instructed to indicate the gender of the presented face by pressing buttons of magnet-compatible button boxes attached to their legs. During the control-condition, participants were instructed to press the button corresponding to the direction of an arrow presented over the scrambled face. Reaction time and accuracy were recorded. The task was presented using E-prime software (Psychology Software Tools, Pittsburgh, PA). Images were projected onto a translucent screen, which was visible for participants by means of a mirror above their head. Average duration of the task was 11.8 minutes.

Analysis behavioral data

Behavioral data of the Faces task were processed using custom-written scripts in Matlab (Mathworks). Data were filtered for each participant by removing trials with reaction times ≤ 300 ms and trials with reaction times more than three standard deviations apart from the mean individual reaction time, following the procedure described by (38). This filtering procedure was applied to remove extreme outlier trials from the subsequent analysis of performance, and eliminated on average 13.21% of trials (CD-patients: 13.24%; controls: 13.18%; no difference between groups (independent-samples t-test: $t(53)=0.06$, $P=0.94$)). Participants with a percentage of missing trials exceeding the upper bound of the 95% confidence interval for the mean percentage of missing trials for each group were excluded. This was the case for two CD-patients and three controls. Subsequently, performance (accuracy and reaction time) was determined for each participant, for all trials and for particular task conditions (scrambled faces; facial expressions; separate for the five different facial expressions: angry, fearful, happy, neutral and sad). Participants with an overall accuracy ≤ 80% (one CD-patient; five controls) were excluded from the dataset. After matching on age, gender and education, 21 CD-patients and 21 controls formed the final sample of this study.

Statistical analyses of performance were performed using IBM SPSS Statistics for Windows (Version 21.0. Armonk, NY: IBM Corp). Repeated measures ANOVAs with condition (facial expressions vs scrambled faces) as within-subjects factor and group (CD-patients vs controls) as between-subjects factor were used to investigate group differences in performance (ac-

curacy and reaction time). In addition, repeated measures ANOVAs with facial expression (angry, fearful, happy, neutral, sad) as within-subjects factor and group (CD-patients vs controls) as between-subjects factor were used to examine whether facial expression influenced performance of the groups. Significance level was set at $P \leq 0.05$.

MRI data acquisition

Imaging data were collected using a Philips 3.0T Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel SENSE (Sensitivity Encoding) head coil and located at the Leiden University Medical Centre. During the Faces task, functional scans were acquired using T2* weighted echo-planar imaging (repetition time (TR)=2200 ms, echo time (TE)=30 ms, 38 axial slices, descending acquisition, 2.75 mm x 2.75 mm x 2.75 mm + 10% interslice gap, field of view 220 x 115 x 220 mm). The first two volumes of the scan were dummy scans and were removed to allow for equilibration of T1 saturation effects. A 3D T1-weighted anatomical scan and a high-resolution EPI-image were acquired for within-subject registration purposes (T1 scan: TR=9.734 ms, TE=4.59 ms, flip angle = 8°, 140 slices, 0.875 x 0.875 x 1.2 mm, FOV = 224 x 168 x 177.333 mm; EPI high-resolution scan: TR = 2200 ms, TE = 30 ms, flip angle = 80°, 84 axial slices, 1.964 x 1.964 x 2 mm). The task was part of a larger scanning session (17-19).

fMRI data analysis

Data analysis was performed using FEAT (FMRI Expert Analysis Tool; version 6.00) (39,40). Pre-statistics processing consisted of motion correction, slice-timing correction using Fourier-space time-series phase-shifting, non-brain removal, spatial smoothing using a Gaussian kernel of FWHM 6.0 mm, grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses, and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with σ=30.0 s). Functional scans of each participant were registered to the individual high-resolution EPI-image, which was in turn registered to the individual 3D T1-weighted anatomical scan and subsequently registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm). Next, event-related statistical analysis of the time-series was carried out in native space. For each participant, six explanatory variables with their temporal derivatives were included in the general linear model, representing the presentation of 1) a scrambled face, 2) an angry face, 3) a fearful face, 4) a happy face, 5) a neutral face and 6) a sad face. Each EV had a duration of 2.5 s and was convolved with a double gamma haemodynamic response function. Subsequently, six contrasts of interest were defined: 1) all emotional faces > scrambled faces (referred to as "viewing faces", representing main effect of task); 2) angry faces > scrambled faces; 3) fearful faces > scrambled faces; 4) happy faces > scrambled faces; 5) neutral faces > scrambled faces and 6) sad faces > scrambled faces. We verified whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5

mm. Subsequently, the individual contrast images were submitted to higher-level mixed-effects group analyses.

Whole-brain analysis: main effect of task

A whole-brain analysis was performed to determine activity related to the main effect of task (emotional faces > scrambled faces). Clusters were tested for significance using a height threshold of z>2.3 and a cluster-corrected significance threshold of P<0.05 (41).

Region Of Interest (ROI) analyses: differences between groups

Given our a priori hypotheses about the amygdala and mPFC, we applied a ROI approach in order to maximize statistical power to detect differences in brain activation between the CD-patients and controls in these areas. To that aim, we performed two separate higher-level analyses, restricted respectively to the amygdala and the mPFC-OFC by applying pre-threshold masking. Masks were created in standard space with a resolution of 2x2x2 mm. The amygdala ROI was defined using the Harvard-Oxford Subcortical Structural Atlas implemented in FSLView (version 3.2.0) and consisted of voxels with a probability of at least 50% of belonging to the left or right amygdala (total size of mask: 505 voxels). The mPFC-OFC ROI was created using the Harvard-Oxford Cortical Structural Atlas implemented in FSLView (version 3.2.0) and consisted of voxels with a probability of at least 10% of belonging to the subcallosal cortex, the frontal medial cortex or the frontal orbital cortex. In addition, we included those voxels of the anterior division of the cingulate gyrus (probability threshold: 10%) positioned anterior of the y-coordinate of 30 mm (total size of mask: 10738 voxels). Masks are shown in Figure 1.

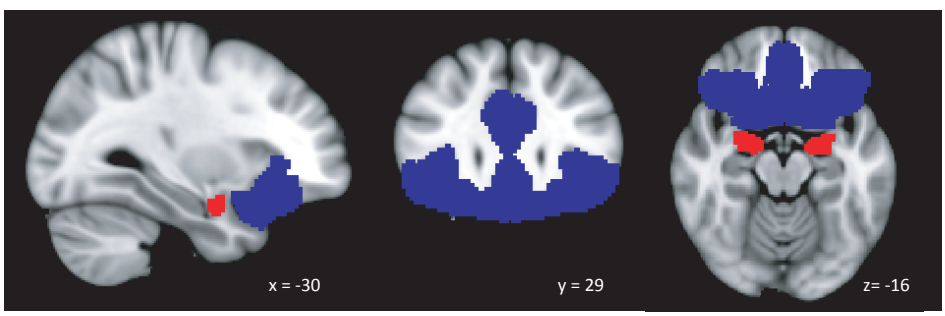

Figure 1. Regions of interest: medial prefrontal – orbitofrontal cortex (mPFC-OFC) (blue; 10738 voxels) and amygdala (red; 505 voxels), superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain.

Within these masks, we investigated activation related to the main effect of task (emotional faces > scrambled faces) for each group separately, and report regions with a height threshold of z>2.3 and a cluster-corrected significance threshold of P<0.05 (41). Subsequently, we

investigated group-differences related to the main effect of task. Significant group-differences within the ROIs were further explored by extracting individual mean z-scores from the lower-level contrast images of the contrast 'emotional faces > scrambled faces' with the Featquery-tool (implemented in FSL 5.0.4). Correlation analyses on these individual z-scores were performed using Pearson's r or, when data violated assumptions for parametric tests, with Kendall's tau. In addition, individual mean z-scores for the other contrasts (each facial expression > scrambled faces) were extracted from significant clusters.

Whole-brain analysis: differences between groups

In addition to the ROI analyses, we performed an exploratory whole-brain analysis to investigate task-related group-differences in brain activation outside the predefined ROIs. Clusters were tested for significance using a height threshold of $z>2.3$ and a cluster-corrected significance threshold of $P<0.05$ (41).

Post-hoc exploratory psychophysiological interaction (PPI) analyses

In order to investigate whether group-differences in activation were accompanied by differences in functional connectivity specific to the task, we performed exploratory psychophysiological interaction (PPI) analyses for significant clusters resulting from our ROI analyses. A PPI analysis investigates whether a task-condition (psychological component) influences the co-variation in activity (physiological component) between a certain seed region and other brain regions (25). For each individual, a first-level PPI analysis was performed using FEAT. Individual timecourses (physiological component) were extracted from a seed region based on the significant cluster obtained in the ROI-analyses. The psychological regressor denoted the task-condition of interest (emotional faces > scrambled faces). We convolved this regressor with a double gamma haemodynamic response function, applied temporal filtering and added a temporal derivative. The product of the demeaned physiological regressor and the zero-centered psychological regressor represented the PPI regressor of interest. In addition, a confound regressor (faces + scrambled faces) was included in the model, to explain shared variance between trial types (regressor convolved with a double gamma haemodynamic response function, temporal filtering applied and a temporal derivative added). Higher-level PPI analyses were used to test for differences between groups in task-related functional connectivity. In order to avoid false-positive results in this exploratory analysis, clusters were tested for significance using a rather stringent height threshold of $z>3.1$ and a cluster-corrected significance threshold of $P<0.05$ (41). Significant clusters were further investigated by extracting the individual z-scores using the Featquery-tool.

RESULTS

Participants

Characteristics of CD-patients and matched healthy controls are presented in Table 1. Patients had a mean estimated duration of disease of 8.2 years (standard deviation (SD): 8.5; range 0.8 – 37.0 y), while the mean duration of remission at the time of evaluation was 10.8 years (SD: 7.9; range 1.9 – 10.8 y). CSI scores during active disease and at the time of evaluation were 8.3 (SD: 2.0, range 5.0-12.0) and 2.5 (SD: 1.6, range 0.0 – 5.0), respectively. Eleven patients (52%) received hydrocortisone replacement. CD-patients had more depressive symptoms than controls on the MADRS ($P<0.001$). Scores on the other questionnaires were not different between groups when correcting for multiple comparisons (Bonferroni-corrected significance level: $P<0.005$; Table 1). However, when a less conservative threshold ($P<0.05$) was applied, patients appeared to have more depressive symptoms as measured with the IDS self-report questionnaire ($P=0.017$) and higher anxiety levels as assessed by both the BAI ($P=0.008$) and the FQ ($P=0.007$). Specifically, patients reported increased levels of social anxiety ($P=0.006$), while scores on agoraphobia and blood-injury phobia did not differ between groups. In addition, patients had higher scores on the Apathy Scale ($P=0.006$) and mentioned higher levels of cognitive failure ($P=0.023$) (Table 1). Patients with and without hydrocortisone replacement did not differ on any of these scores (data not shown).

Behavioral data

Performance scores (accuracy and reaction time) on the Faces task are presented in Figure 2 (see for detailed scores Supplementary Table 1). There were no group-differences with respect to the percentage of missing trials (i.e. trials in which no response was given; independent-samples t-test: $t(40)=-1.06$, $P=0.29$). Repeated Measures ANOVAs (condition (facial expressions vs scrambled faces) x group) revealed a significant effect of condition on both accuracy ($F(1,40)=4.09$, $P=0.05$) and reaction time ($F(1,40)=9.89$, $P=0.003$). However, there was no effect of group on performance (accuracy: $F(1,40)=0.01$, $P=0.91$; reaction time: $F(1,40)=0.13$, $P=0.72$) nor an interaction between group and condition (accuracy: $F(1,40)=0.20$, $P=0.66$; reaction time: $F(1,40) = 3.02$, $P=0.09$). Post-hoc paired-sample t-tests revealed that all participants reacted slower ($t(41)=3.1$, $P=0.004$), but were more accurate ($t(41)=-2.0$, $P=0.047$) in trials with facial expressions compared to trials with scrambled faces.

Additional repeated measures ANOVAs investigating the effect of facial expression on performance (facial expression x group) revealed no significant effect of facial expression on either accuracy ($F(4,160)=1.64$, $P=0.17$) or reaction time ($F(4,160)=1.17$, $P=0.33$). In addition, there was no significant effect of group (accuracy: $F(1,40)=2.84$, $P=0.10$; reaction time: $F(1,40)=1.30$, $P=0.26$) nor a significant interaction between facial expression and group (accuracy: $F(4,160)=0.58$, $P= 0.68$; reaction time: $F(4,160)=0.17$, $P=0.95$) (Figure 2).

Table 1 Characteristics of participants and results of psychopathology and cognitive performance. Data are presented as mean ± standard deviation or as numbers

	CD-patients (n =21)	Healthy controls (n=21)	P value
Age (years)	45.0 ± 7.9	45.9 ± 6.7	0.693 [a]
Gender (male/female)	4 / 17	4 / 17	1.000 [b]
Education (n)			
Low	5	5	1.000 [b]
Medium	10	10	1.000 [b]
High	6	6	1.000 [b]
Surgery (n)			
Transsphenoidal adenomectomy	21 (100%)		
Bilateral adrenalectomy	1 (5%)		
Radiotherapy (n)	5 (24 %)		
Disease duration (years)	8.2 ± 8.5		
Duration of remission (years)	10.8 ± 7.9		
Clinical severity index (total score)			
Active phase	8.3 ± 2.0		
Remission phase	2.5 ± 1.6		
Hydrocortisone substitution (n) Mean dose: 18.4 mg/day (± 10.3 mg)	11 (52 %)		
Montgomery-Åsberg Depression Rating Scale (MADRS)	5.9 ± 5.7	1.3 ± 1.5	0.000 [c] **
Inventory of Depression Symptomatology (IDS) #	46.2 ± 13.8	34.9 ± 3.3	0.017 [c] *
Beck Anxiety Inventory (BAI) #	28.2 ± 6.1	23.6 ± 2.8	0.008 [c] *
Fear Questionnaire (FQ) #			
Total Score	26.8 ± 17.5	13.6 ± 10.5	0.007 [a] *
Agoraphobia Subscale	6.8 ± 8.4	2.9 ± 4.9	0.242 [c]
Blood Injury Phobia Subscale	6.8 ± 8.9	3.5 ± 4.4	0.327 [c]
Social Phobia Subscale	13.2 ± 7.8	7.2 ± 5.1	0.006 [a] *
Apathy Scale (AS) #	13.2 ± 6.7	8.2 ± 3.8	0.006 [a] *
Irritability Scale (IS) #	11.7 ± 9.5	7.3 ± 4.8	0.147 [c]
Cognitive Failures Questionnaire (CFQ) #	37.9 ± 17.8	25.4 ± 8.0	0.023 [c] *

Differences between groups were tested with an independent-samples t–test ([a]), χ^2 –test ([b]) or Mann-Whitney U test ([c]).

[#]: Scores based on data from 20 CD patients.

*: Group-difference at uncorrected significance level P <0.05.

**: Group-difference at Bonferroni-corrected significance level P < 0.005.

Whole-brain analysis: main effect of task

The whole-brain analysis revealed task-related (emotional faces > scrambled faces) activation clusters in line with those reported in earlier work (34–36). In both patients and controls, significant brain activation was present in the bilateral occipital cortex and fusiform gyrus, bilateral amygdala and hippocampus, and several prefrontal areas (Figure 3A and 3B; Supplementary Table 2).

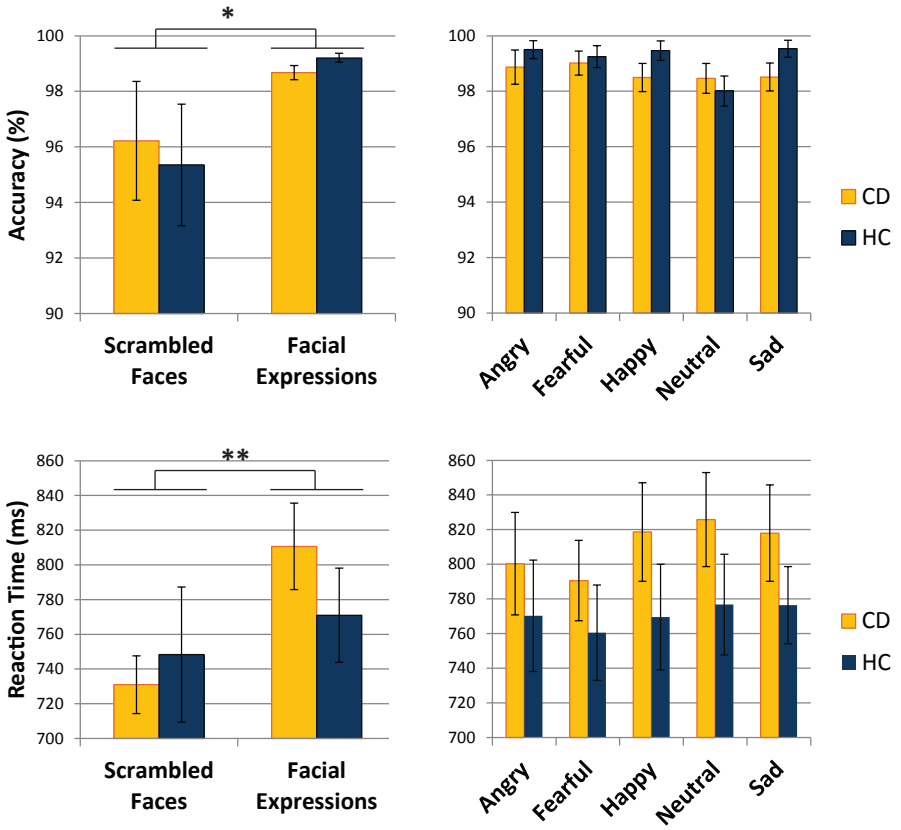

Figure 2. Performance on Faces task. Error bars represent standard error of the mean. *: P<0.05, **: P<0.005.

Region Of Interest (ROI) analyses: differences between groups

Amygdala

In line with our hypothesis and the results of the whole-brain analysis, viewing faces was associated with significant bilateral amygdala activation, both in patients with CD and controls (Supplementary Figure 1; Supplementary Table 3). There was, however, no significant difference in brain activation levels between groups at the predefined threshold (z>2.3, cluster-corrected significance threshold of P<0.05). Also when we applied a more liberal voxel threshold of P<0.01 (uncorrected), no significant activation differences between groups emerged.

mPFC-OFC

Within the predefined mPFC-OFC ROI, viewing faces was associated with activation in bilateral orbitofrontal cortex in both groups (Supplementary Figure 2; Supplementary Table 4).

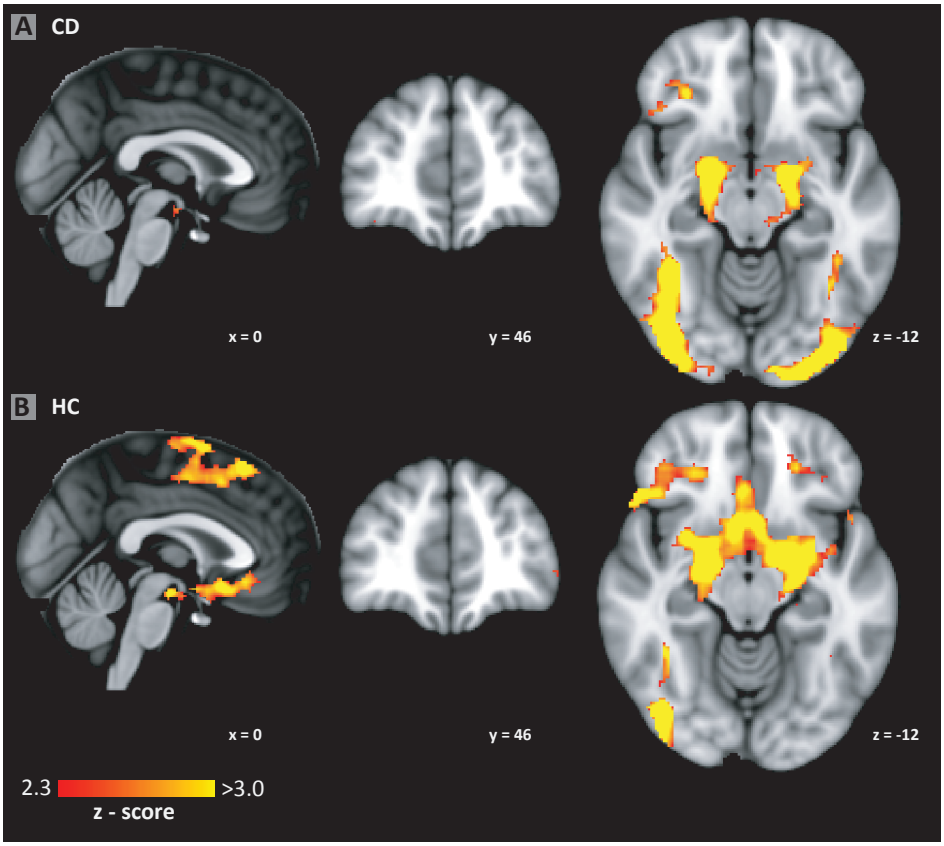

Figure 3. Significant activation clusters related to viewing faces (emotional faces > scrambled faces) at whole-brain level in CD-patients with long-term remission (CD; Figure 3A) and control participans (HC; Figure 3B). Significant clusters are superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain.

Group-comparisons showed decreased activation of the ventromedial PFC (vmPFC) in CD-patients relative to controls (clustersize: 274 voxels, P=0.021; Figure 4A and Supplementary Table 4). To further illustrate the group-difference in brain activation within the vmPFC, mean z-scores were extracted from the lower-level contrast images for each individual, using a mask including those voxels showing a significant group-difference (z>2.3) within the vmPFC ROI (from now on referred to as 'vmPFC group-difference mask'). Results are presented in Figure 4B.

In addition, the vmPFC group-difference mask was used to extract individual mean z-scores for the five lower-level contrasts representing activation related to the separate facial expressions (angry, fearful, happy, neutral, sad) relative to scrambled faces, in order to investigate whether the difference in vmPFC activation between the groups was influenced by specific facial expressions. A repeated measures ANOVA (facial expression x group) con-

firmed the effect of group (F(1,40)=8.65, P=0.005), but revealed no effect of facial expression (F(4,160)=1.61, P=0.19); in addition, there was no interaction between facial expression and group (F(4,160)=0.57, P=0.65) (Figure 4C). These findings indicate that the vmPFC-hypoactivation in CD-patients is not driven by viewing a face with a specific emotional expression, but rather represents an overall effect of viewing faces.

Relation between vmPFC activation and clinical characteristics

Within the group of patients, there was no relation between activation (contrast emotional faces > scrambled faces) in the vmPFC cluster and scores on the MADRS (Kendall's tau=0.22,

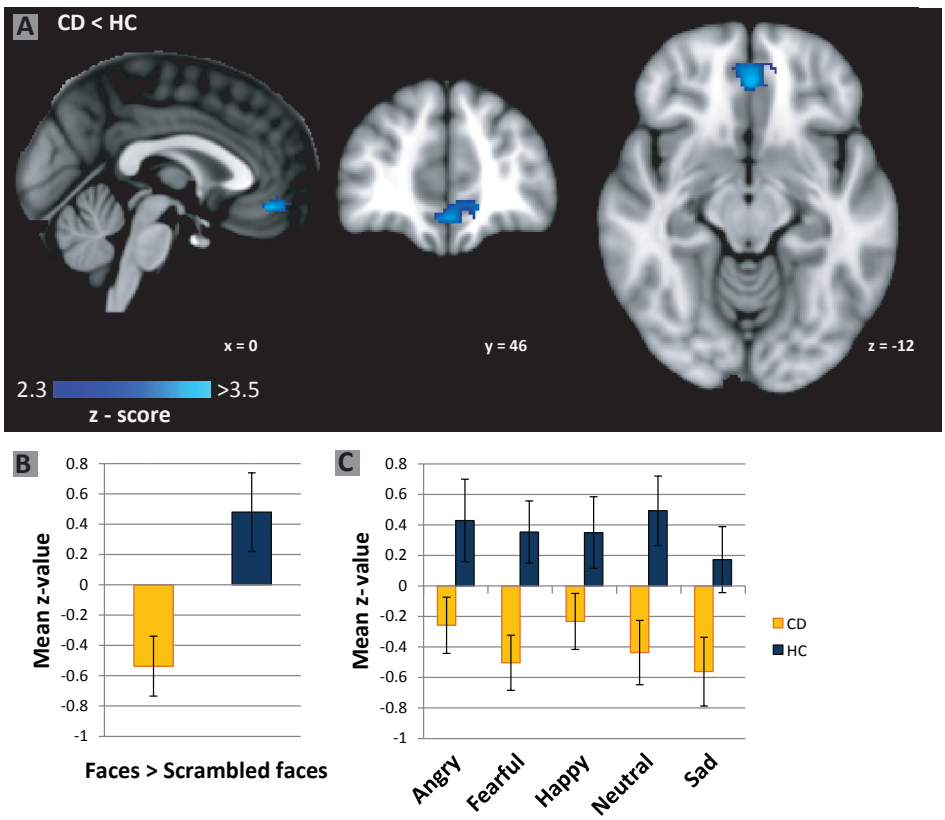

Figure 4. Significant group difference in activation related to viewing faces (emotional faces > scrambled faces) within the mPFC-OFC ROI (Figure 4A). CD-patients with long-term remission (CD) had significantly lower activation levels in the vmPFC when compared to control participants (HC). Significant clusters are superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain. Blue colors indicate decreased activation levels in CD-patients relative to control participants. For illustrative purposes, individual z-scores were extracted from the vmPFC cluster and presented in Figure 4B (emotional faces > scrambled faces) and Figure 4C (separate facial expressions). Analyses confirmed significantly decreased activation levels in CD-patients with long-term remission compared to controls, but no effect of facial expression.

P=0.18). In addition, there was no difference in vmPFC activation levels between patients who received radiotherapy (n=5) and patients who did not receive radiotherapy (n=16; t(19) =-0.38, P=0.71), neither was there a difference between patients with hydrocortisone replacement (n=11) and those who were not glucocorticoid dependent (n=10; t(19)=-1.5, P=0.15). Furthermore, there was no relation between vmPFC activation and disease duration (Kendall's tau=0.13, P=0.40) or the severity of symptoms as measured with the CSI during active disease (Pearson's r=-0.03, P=0.89) or during remission (Kendall's tau = 0.15, P=0.37). The relation between the duration of remission and vmPFC activation was significant at trend level (Pearson's r =-0.41, P= 0.06), indicating that a longer duration of remission is associated with more activation in the vmPFC.

Whole-brain analysis: differences between groups

Comparison of whole-brain activation levels (contrast emotional faces > scrambled faces) between the groups revealed decreased brain activation in CD-patients relative to controls in a cluster in the mPFC (Supplementary Table 2). This cluster extended into the left frontal pole (clustersize: 578 voxels, P=0.021). There were no clusters where CD-patients showed increased activation relative to controls.

Post-hoc exploratory PPI analyses

We performed a post-hoc exploratory PPI analysis with the hypoactive cluster in the vmPFC as seed region. Using the vmPFC group-difference mask, we extracted for each participant the mean time course from this region. This time course constituted the physiological regressor in the model, while the psychological regressor denoted the task-condition of interest (emotional faces > scrambled faces). Comparison of the groups revealed no significant clusters within the amygdala ROI, indicating that groups did not differ in task-related func-

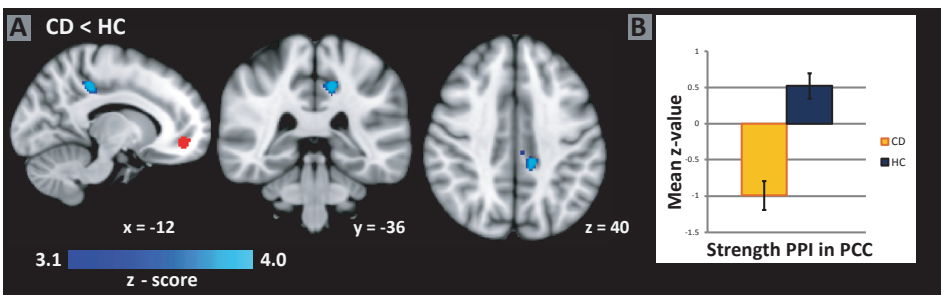

Figure 5. Significant group difference in functional connectivity during the faces vs scrambled task condition (PPI) between the vmPFC (red) and the posterior cingulate cortex (PCC; blue colors indicate decreased functional connectivity in CD- patients relative to control participants) (Figure 5A). CD-patients with long-term remission had significantly decreased positive functional coupling when compared to control participants (HC). For illustrative purposes, individual z-scores were extracted from the PCC cluster and presented in Figure 5B. Significant clusters superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain.

tional connectivity between the vmPFC cluster and the amygdala. Exploratory analyses at the whole-brain level revealed a significant group difference in task-condition associated changes in connectivity at the whole brain level (Figure 5): CD-patients showed a decreased positive functional coupling related to the task (emotional faces > scrambled faces) between the vmPFC cluster and the posterior cingulate cortex (PCC) relative to controls (cluster size: 139 voxels, P=0.049; maximum z-value 4.19 at MNI coordinate (X,Y,Z) -12, -34, 42).

DISCUSSION

The present study is the first to demonstrate task-related functional brain abnormalities in patients with long-term remission of CD relative to matched healthy control participants. We found hypoactivation of the ventromedial prefrontal cortex (vmPFC) during processing of facial expressions (versus scrambled faces), without alterations in amygdala activation. This vmPFC hypoactivation was not elicited by a specific facial expression. The post-hoc exploratory psychophysiological interaction (PPI) analysis, investigating task-related correlations in brain activity between regions, revealed decreased functional coupling between the vmPFC and the posterior cingulate cortex (PCC). These functional brain abnormalities may, at least in part, underlie the long-term psychological morbidity as is observed after successful correction of hypercortisolism (4).

The vmPFC hypoactivation reported here (Figure 3 and Figure 4) is in line with the results of earlier work on prefrontal functioning during emotional processing in stress-related disorders. The vmPFC is functionally and structurally connected to the amygdala and is, due to its role in fear learning and fear extinction, implicated in the pathogenesis of anxiety and mood disorders (22). Decreased mPFC activation has been reported during emotional tasks in patients with specific phobia and panic disorder (42), women with generalized anxiety disorder (43), men with posttraumatic stress disorder (PTSD) (24) and individuals reporting emotional maltreatment during their childhood (44). However, the present results are in striking contrast with earlier findings in patients with active CD, since patients with active disease demonstrated higher activation levels in frontal, medial, and subcortical regions during the identification of emotional faces (12). It should, however, be noted that this hyperactivation was accompanied by lower accuracy in task performance in patients compared to controls (12), while we did not find any group differences in task performance in our study (Figure 2; Supplementary Table 1). These findings tentatively suggest that correction of hypercortisolism may induce a switch in vmPFC activation levels from hyperactivation to hypoactivation in response to emotional stimuli. Future longitudinal research is needed to study the time course of dynamic changes that occur after correction of hypercortisolism.

We speculate that the hypoactivation in the vmPFC, in combination with the earlier described alterations in brain structure and white matter integrity in the same sample of patients (17,18), underlies the persisting psychopathology previously reported in patients with

long-term remission of CD (2,7). In line with these results, the patient sample investigated here showed an increased prevalence of depressive symptoms, anxiety levels and symptoms of apathy (Table 1). Although patients had clear symptoms and differed significantly from controls, it should be noted that their scores on depression scales were still within the normal range (26), indicating that the patients would not have been classified as having a clinical depression. This observation suggests that the functional brain changes in patients with long-term remission of CD reported here are not the result of suffering from a depressive disorder. Alternatively, this null-effect could be caused by insufficient power due to the relatively small sample size or reflect the limited sensitivity of questionnaires to measure specific elements of psychopathology. Furthermore, it is possible that the hypoactivation of the vmPFC only enhances the sensitivity to develop psychopathology, but that the actual expression of psychopathology is also dependent on other factors, such as environmental factors and personality traits. Future studies with a larger sample size could provide more insight into this relationship, as well as into the influence of other factors such as duration of remission or glucocorticoid dependency. These factors showed weak and insignificant relationships with vmPFC activation in our study, but are potentially confounding factors that should be taken into account.

As expected, we observed robust amygdala activation in response to facial expressions (Supplementary Figure 1). Contrary to our hypothesis, we did not find any group differences in amygdala activation. We expected amygdala hyperactivation in this sample of patients compared to healthy controls, based on studies reporting increased amygdala activation in patients with other stress-related disorders, such as PTSD (45), social anxiety disorder (SAD) (46), and participants with previous childhood maltreatment (35); for a review see (21). However, our results coincide with those from other studies reporting no difference in amygdala activation levels between patients and healthy controls, for example in women with generalized anxiety disorder (43), patients with generalized SAD (47) and patients with depression and anxiety (36). The absence of alterations in amygdala activity might also relate to the specifics of our task-paradigm, since the results of various experiments, reviewed by (22), suggest that task-related factors like the amount of attention and the level of awareness of the stimuli could possibly influence the level of amygdala activation in anxiety. However, more research is needed to explore this relationship between task specifics and amygdala activation levels into more detail. Alternatively, the lack of detection of a group difference in amygdala activation could possibly be caused by differences in baseline amygdala blood flow and blood oxygenation level-dependent (BOLD) responses between patients and controls, which could not be measured with the methods used in this study.

Additional post-hoc PPI analyses revealed decreased functional coupling in patients between the vmPFC and the posterior cingulate cortex (PCC) during the task-condition (emotional faces > scrambled faces) (Figure 5). The PCC is suggested to play an important role in directing attention, regulating cognition, memory-related processes and self-referential

tasks, but it's exact function is still a matter of debate (48). The mPFC and PCC are structurally connected by the cingulum tract and are functionally connected as part of the so-called default mode network (DMN) (49,50). Earlier research by our group showed decreased white matter integrity in the cingulum tract in the same group of CD patients (18). Here, we expand this finding by showing decreased functional coupling between the areas that are structurally connected by the cingulum tract. To the best of our knowledge, we are the first to report on changes in task-condition associated functional connectivity in patients with CD after long-term remission. Although this finding is preliminary due to the exploratory nature of the analysis, it is in line with the results of earlier work on stress-related disorders. For example, a recent review reported decreased functioning of the DMN in patients with anxiety disorders in tasks in which they were supposed to regulate their emotions without explicit instructions (51). In addition, Bluhm and colleagues showed decreased connectivity between the mPFC and the PCC in women with PTSD (52). However, these studies determined functional connectivity during rest, while we observed changes in task-condition dependent functional connectivity. Future research is needed to elaborate on the aberrant functional coupling between the vmPFC and the PCC in patients with long-term remission of CD, in order to investigate the direction of these changes.

The findings of this study provide new insight into the functional brain alterations underlying the persisting psychological morbidity seen in patients after treatment of CD, who have always been considered cured after correction of cortisol excess. Although no formal conclusions can be drawn about causal relations between the effects of previous exposure to excessive cortisol levels and functional brain alterations given the cross-sectional design of this study, the results presented here strongly suggest that hypercortisolism induces persistent changes in brain activation. The use of a longitudinal design in future research enables to provide more insight into the course of the described functional abnormalities.

In conclusion, the present study clearly shows long-term changes in brain activation patterns in patients with CD despite long-term remission of cortisol excess. Thereby, we have provided insight in the effects of past hypercortisolism on the brain. These functional alterations may, together with the previously reported structural abnormalities in cerebral grey and white matter, underlie the long-term psychological morbidity in patients with CD after correction of hypercortisolism. Prospective studies with a long-term follow-up could provide more insight into the longitudinal changes that occur after correction of cortisol excess.

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Supplementary Table 1. Performance on Faces Task

	CD-patients (n =21)	Healthy controls (n=21)	P-value [a]
Missings (%)	3 ± 2	3 ± 2	0.29
Accuracy (%)			
Scrambled faces	96 ± 10	95 ± 10.0	0.78
Facial expressions	99 ± 1	99 ± 1	0.09
Angry	99 ± 3	100 ± 2	
Fearful	99 ± 2	99 ± 2	
Happy	99 ± 2	100 ± 2	
Neutral	98 ± 3	98 ± 3	
Sad	99 ± 2	100 ± 1	
Reaction time (ms)			
Scrambled faces	726 ± 76	742 ± 178	0.71
Facial expressions	807 ± 114	766. ± 124	0.27
Angry	798 ± 135	764 ± 147	
Fearful	793 ± 106	755 ± 126	
Happy	814 ± 130	763 ± 140	
Neutral	817 ± 125	772 ± 133	
Sad	814 ± 127	772 ± 102	

All presented scores are mean ± standard deviation. Differences between groups were tested with an independent-samples t-test ([a]).

Supplementary Table 2. Activation clusters (whole-brain) in response to emotional faces (vs scrambled faces)

		MNI coordinates peak voxel					
	Region	**X**	**Y**	**Z**	**Max. Z value**	**# voxels**	**P-value**
HC	Bilateral amygdala and hippocampus	20	-4	-16	5.41	6729	<0.001
	Right IFG, extending into right MFG	52	22	22	4.67	4118	<0.001
	Bilateral SFG	8	14	52	4.45	1814	<0.001
	Right Temporal Occipital Fusiform Cortex, extending into LOC	38	-50	-22	6.17	1023	<0.001
	Left Temporal Fusiform Cortex, extending into ITG	-38	-42	-26	5.39	623	0.014
	Postcentral Gyrus	56	-12	46	3.38	495	0.045
CD	Right MFG, extending into right IFG	54	26	30	4.7	2880	<0.001
	Bilateral amygdala and hippocampus	20	-4	-16	5.25	2699	<0.001
	Right Occipital Fusiform Gyrus, extending into Temporal Occipital Fusiform Cortex	36	-78	-12	5.46	2627	<0.001
	Left Temporal Fusiform Gyrus, extending into Occipital Fusiform Cortex	-38	-44	-22	5.69	1664	<0.001
	Left IFG, extending into left MFG	-44	16	28	4.08	748	0.005
HC > CD	mPFC, extending into left Frontal Pole	-22	40	-12	3.37	578	0.021

There were no activation clusters CD > HC.
HC: healthy controls; CD: patients with long-term remission of Cushing's Disease.
IFG: Inferior Frontal Gyrus; MFG: Middle Frontal Gyrus; SFG: Superior Frontal Gyrus; LOC: Lateral Occipital Cortex; ITG: Inferior Temporal Gyrus; mPFC: medial Prefrontal Cortex.

Supplementary Table 3. Activation clusters within amygdala ROI in response to emotional faces (vs scrambled faces)

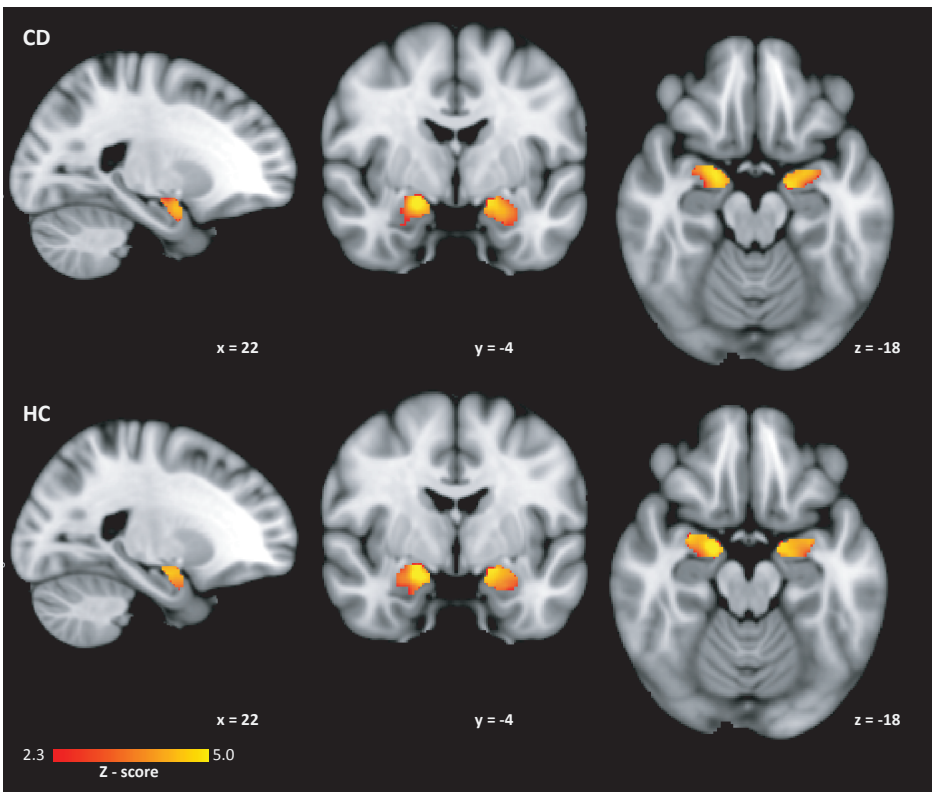
		MNI coordinates peak voxel					
	Region	**X**	**Y**	**Z**	**Max. Z value**	**# voxels**	**P-value**
HC	Right amygdala	20	-4	-16	5.41	256	0.002
	Left amygdala	-18	-4	-14	4.86	219	0.002
CD	Right amygdala	20	-4	-16	5.25	248	0.002
	Left amygdala	-16	-6	-16	4.94	206	0.003

There were no group differences in activation levels within this ROI.
HC: healthy controls; CD: patients with long-term remission of Cushing's Disease.

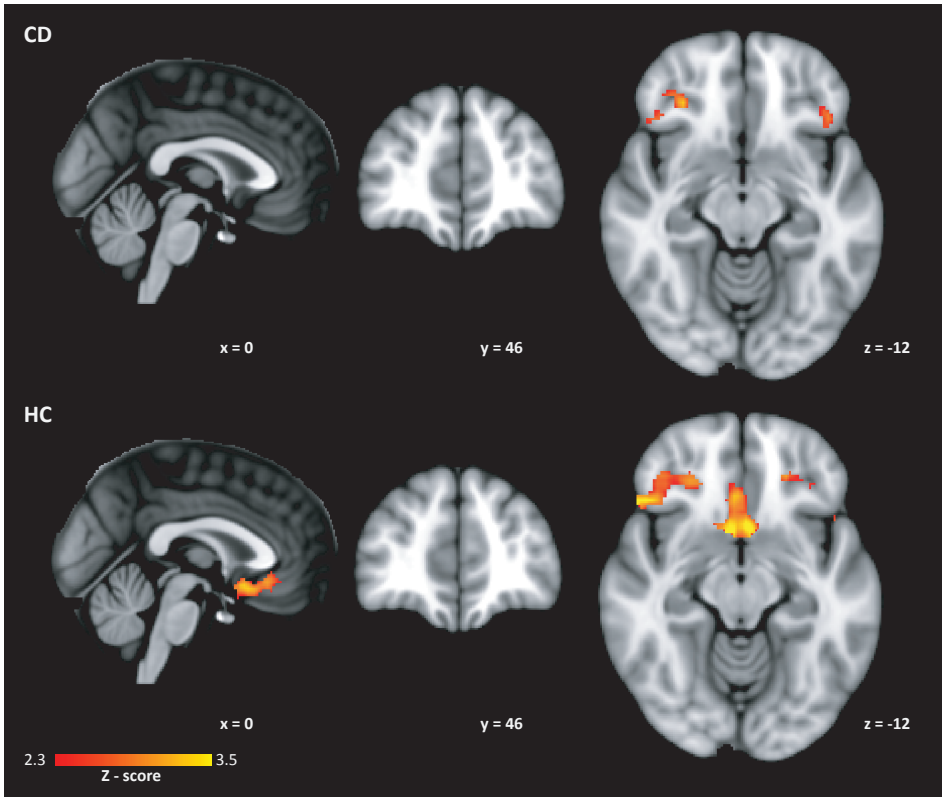
Supplementary Table 4. Activation clusters within mPFC ROI in response to emotional faces (vs scrambled faces)

		MNI coordinates peak voxel					
	Region	**X**	**Y**	**Z**	**Max. Z value**	**# voxels**	**P-value**
HC	Bilateral OFC, extending into subcallosal cingulate gyrus	28	6	-16	4.07	926	< 0.001
	Right Insular Cortex	36	22	0	4.26	769	< 0.001
	Left Insular Cortex	-30	26	4	3.94	277	0.020
CD	Right OFC, extending into right IFG	48	26	-2	4.04	498	0.002
	Left OFC	-38	32	-16	3.36	213	0.044
CD < HC	vmPFC	0	42	-12	3.2	274	0.021

There were no activation clusters CD > HC within this ROI.
HC: healthy controls; CD: patients with long-term remission of Cushing's Disease.
OFC: Orbitofrontal Cortex; IFG: Inferior Frontal Gyrus; vmPFC: ventromedial Prefrontal Cortex.



Supplementary Figure 1. Significant activation clusters related to viewing faces (emotional faces > scrambled faces) within the amygdala ROI. There were no differences in activation levels between CD-patients with long- term remission (CD) and control participants (HC). Significant clusters are superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain.



Supplementary Figure 2. Significant activation clusters related to viewing faces (emotional faces > scrambled faces) within the mPFC ROI. Significant clusters are superimposed on the template MNI_T1_152_1mm_brain. All images are displayed according to radiological convention: right in image is left in brain.