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## Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres



### Decreased hair cortisol concentrations in generalised anxiety disorder

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#### ARTICLE INFO

#### Article history: Received 3 December 2009 Received in revised form 2 June 2010 Accepted 6 September 2010

Keywords: Hypocortisolism Retrospective assessment Diurnal Saliva

#### ABSTRACT

Previous research examining hypothalamic–pituitary–adrenal (HPA) axis activity in generalised anxiety disorder (GAD) has suggested a general hypercortisolism. These studies have mostly relied on salivary, plasma or urinary assessments, reflecting cortisol secretion over short time periods. The current study utilised the novel method of cortisol assessment in hair to obtain a retrospective index of cortisol secretion over a prolonged period of time. Hair cortisol levels were determined in 15 GAD patients and in 15 age- and gendermatched controls. In addition, participants collected six saliva samples (on awakening, +30 min, 12:00, 16:00, 20:00 h and at bedtime) on two consecutive weekdays for the assessment of the diurnal cortisol profile. Results revealed significantly lower (50–60%) cortisol levels in the first and second 3-cm hair segments of GAD patients compared to those of controls. No significant between-group differences were seen in diurnal cortisol profiles. The hair cortisol findings tentatively suggest that under naturalistic conditions GAD is associated with hypocortisolism. If corroborated by future research, this demonstrates the important qualities of cortisol measurement in hair as an ecologically valid, retrospective index of long-term cortisol secretion and as a marker for psychiatric disorders associated with hypo- or hypercortisolism.

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#### 1. Introduction

The hypothalamus-pituitary-adrenal (HPA) axis with its peripherally active hormone cortisol constitutes an important neuroendocrine response system. Altered activity of the HPA axis as a result of chronic stress has been considered to play a role in the aetiology of several psychiatric disorders (Miller et al., 2007). An important condition in this respect is generalised anxiety disorder (GAD), which is characterized by excessive anxiety and uncontrollable worry about various life problems and circumstances that occur on the majority of days for at least 6 months (American Psychiatric Association, 2000). Although these features of GAD suggest that suffering from this condition might involve repeated stressful experiences which over time could lead to an altered cortisol secretory pattern, little research to date has examined the neuroendocrine changes underlying GAD.

The currently available evidence on HPA axis functioning in GAD shows a heterogeneous picture. A number of studies have provided support for the notion that GAD is associated with an upregulation of adrenocortical activity; i.e. hypercortisolism. In two earlier studies, elevated dexamethasone nonsuppression rates were found in GAD patients (Schweizer et al., 1986; Tiller et al., 1988), indicating reduced negative feedback sensitivity of the HPA axis. More recently, Mantella

et al. (2008) showed that GAD patients exhibited an elevated diurnal cortisol profile compared to a healthy control group. In addition, both cognitive therapy (Tafet et al., 2005) and pharmacological treatment (Pomara et al., 2005) of GAD has been reported to be associated with an attenuation of plasma cortisol levels. On the other hand, a number of studies have failed to show aberrant adrenocortical activity in GAD. Rosenbaum et al. (1983) found no differences between 24-h urinary cortisol levels of GAD patients and those of a healthy control group. Similarly, two other studies reported no differences in plasma cortisol levels between GAD patients and healthy controls (Hoehn-Saric et al., 1991) or between patients with major depressive disorder, GAD and control participants (Kelly and Cooper, 1998).

An important factor in the above findings is the assessment of cortisol levels. Whilst the use of salivary, plasma or urinary samples constitute well-established methods, it is important to note that these reflect acutely circulating cortisol levels (*saliva* or *plasma*) or mean cortisol secreted over a somewhat longer period (usually 24 h; *urine*). It can be argued that estimates obtained via these techniques are less well-suited for reflecting normal, long-term cortisol secretion under naturalistic circumstances since the HPA axis is a system which is highly reactive and which shows considerable intra-individual variability (e.g. Hellhammer et al., 2007).

Only recently, a novel technique of assessing endogenous concentrations of cortisol in human hair was introduced (Raul et al., 2004). Importantly, as it is assumed that cortisol is incorporated into the hair shaft during hair growth, the examination of cortisol levels in

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a specific hair segment should provide a retrospective measure of cortisol secretion over the time period during which the hair segment had grown (for a review see Gow et al., 2010; Kirschbaum et al., 2009). This notion has been broadly supported by animal as well as human research. In male rhesus macaques, strong positive correlations between hair cortisol and mean salivary cortisol levels have been reported (Davenport et al., 2006) and in domestic cats and dogs, hair cortisol levels correlated strongly with cortisol concentrations in faeces (Accorsi et al., 2008). In humans, elevated hair cortisol levels were reported in patients with Cushing's syndrome, a condition associated with hypercortisolism (Thomson et al., 2010), and recent work by our group was able to show that the well-established twofold increase in cortisol levels during the third trimester of pregnancy was also reflected in hair segments assumed to have grown over this time period (Kirschbaum et al., 2009). Furthermore, evidence has been reported confirming marker qualities of hair cortisol levels with respect to chronic stress (Davenport et al., 2006; Kalra et al., 2007; Yamada et al., 2007). Finally, whilst some indication was seen that hair cortisol levels in humans show a monotonic decline from scalpnear to more distal hair segments, i.e. are gradually washed out; we have recently provided evidence suggesting that hair analysis can provide a valid retrospective reflection of cortisol secretion for a period of up to six months (Kirschbaum et al., 2009).

The aim of the present study was to compare long-term HPA axis activity in GAD patients and matched healthy controls via the assessment of hair cortisol levels. Despite some heterogeneity in the previous literature, most evidence suggests that GAD is associated with hypercortisolism and hence it was predicted that higher hair cortisol levels would be found in GAD patients than in the control group. To allow a comparison of different methodologies of cortisol assessment, saliva samples for the measurement of a full diurnal cortisol profile were also obtained besides hair samples. Finally, it was investigated whether the previously reported wash out effect was also evident in the current hair samples.

#### 2. Methods

#### 2.1. Participants

Participant recruitment was conducted via local advertisements and flyers. Overall, 95 participants were screened for admission to the study on the basis of health-related questions and the DSM-IV criteria of GAD. 19 participants were then interviewed faceto-face using the Munich-Composite International Diagnostic Interview (M-CIDI; Wittchen and Pfister, 1997), a standardized diagnostic instrument for the assessment of DSM-IV mental disorders. M-CIDI diagnoses were confirmed by an experienced clinical psychologist (KB). Individuals with a current principal or secondary diagnosis of GAD were included in the study. Exclusion criteria were a lifetime diagnosis of substance dependence, psychosis or bipolar disorder. Inclusion to the control group was conducted on the basis that participants were physically healthy, free of medication and had no psychiatric lifetime disorder based on the M-CIDI. In addition, participants of both groups had to have a minimal hair length of 3 cm.

Fifteen GAD patients (13 females, mean  $age \pm standard$  deviation (S.D.):  $36.47 \pm 10.27$ ) and 15 age- and gender-matched control participants (13 females, mean  $age \pm S.D.$ :  $35.67 \pm 9.28$ ) took part in the study. Of the patient group, 11 had received a primary- and 4 a secondary GAD diagnosis. The GAD group showed a range of psychiatric comorbidities, including major depressive disorder (n=11), dysthymic disorder (n=1), panic disorder with or without agoraphobia (n=6), specific phobia (n=6), social phobia (n=5), pain disorder (n=3), obsessive-compulsive disorder (n=1) and alcohol abuse (n=1). Five GAD patients had received psychotherapeutic treatment over the course of their lives. One GAD patient reported suffering from diabetes mellitus and hypertension and another patient reported the use of antidepressant medication.

All participants received a small monetary reward of 10 € and provided written informed consent prior to taking part in the study. The ethics committee of the Carl Gustav Carus clinic of the Technische Universitaet Dresden (Germany) approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki.

#### 2.2. Clinical and psychological measures

Information about sociodemographic variables (sex, age, smoking status, body mass index, use of oral contraceptives and menopausal status), hair-specific characteristics (washes per week, curls, waves, semi-permanent colour, coloration.

permanent wave and natural hair colour) as well as participant health and well-being were obtained using a self-developed questionnaire. In addition to the M-CIDI (Wittchen and Pfister, 1997), the German version of the Penn State Worry Questionnaire (PSWQ; Stöber, 1995) was used to assess the extent of worry. The German version of the Beck Depression Inventory (BDI-II; Hautzinger et al., 2006) was further employed to obtain information about the severity of depressive symptomatology. The Perceived Stress Scale (PSS; Cohen et al., 1983) was used to assess perceived stress over the last month whilst the Trier Inventory for the Assessment of Chronic Stress (TICS; Schulz et al., 2004) provided a chronic stress measure for the last 3 months.

#### 2.3. Sample collection and preparation

#### 2.3.1. Hair cortisol analysis

Hair strands were taken scalp-near from a posterior vertex position and cut into 3-cm segments. Each segment was assumed to represent hair grown over a 3-month period, based on an average hair growth rate of 1 cm per month (Hayashi et al., 1991; Wennig, 2000). Consequently, cortisol concentrations in the first two hair segments would provide a retrospective index of cortisol secretion over the past 6 months. Up to three 3-cm segments were analysed where this was permitted by individuals' hair length.

The wash procedure and steroid extraction was based on the laboratory protocol described in Kirschbaum et al. (2009). Briefly, a minimum of 45 mg of hair was weighed out and each hair segment was put into a 15 ml tube (Falcon, Frickenhausen, Germany) to which 2.5 ml isopropanol was added and the tube was gently mixed on an overhead rotator for 3 min twice. A new wash solvent was added for the second wash cycle. After drying for at least 8 h in a clean protected hood, hair segments were powdered using a Retsch ball mill (Type MM 400, Retsch GmbH, Haan, Germany) for 2 min at 30 Hz. A 25 mg aliquot of powdered hair was carefully weighed out and transferred into a 2 ml cryo vial (Eppendorf, Hamburg, Germany). For steroid extraction, 1.5 ml of pure methanol was added, and the vials were then slowly rotated for 24 h on an overhead rotator. The samples were then spun in a microcentrifuge for 2 min at 10.000 rpm and 1 ml of the clear supernatant was transferred into a fresh 2 ml cryo vial (Eppendorf, Hamburg, Germany). The alcohol was then evaporated under a constant stream of nitrogen at 60 °C. After approximately 20 min, the samples were completely dried and 0.4 ml of phosphate buffer (CAL A, IBL-Hamburg, Germany) added. Finally, the tubes were vortexed for 15 s. For the determination of cortisol,  $100\,\mu l$  from the vial were analysed via a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany).

Seventeen hair samples were processed in duplicate to test the reliability of hair preparation. Following milling of hair, two 25 mg aliquots of powdered hair per segment were processed in parallel. Spearman correlations indicated a high reliability of cortisol measurement in the first and second segment ('r's = 0.957 and 0.991, respectively; both 'P's < 0.001).

#### 2.3.2. Diurnal cortisol profile

Participants collected six saliva samples (on awakening,  $\pm$  30 min, 12:00, 16:00, 20:00 h and at bedtime) on two consecutive weekdays to allow the assessment of a full diurnal cortisol profile. *Salivette* devices (Sarstedt, Rommelsdorf, Germany) were used to obtain saliva samples. Participants were instructed to take nil by mouth other than water and to refrain from smoking for a period of 30 min prior to saliva sampling. They were also told not brush their teeth to avoid abrasion and micro-vascular leakage. Otherwise, participants were free to follow their normal routines. Salivette cotton swabs were stored in *MEMS 6 TrackCap* containers (Aardex Ltd., Switzerland), which electronically detect and store time points when the container is opened and, hence, allow verification of participants' adherence to the sampling regime. Participants also noted down the exact time point for each saliva sample in a diary. Samples were stored at  $\pm$  20 °C in a laboratory freezer. After thawing, saliva samples were centrifuged for 10 min at 4000 rpm. Salivary cortisol concentrations were analysed via a commercially available chemiluminescence assay (CLIA, IBL-Hamburg, Germany).

#### 2.4. Statistic analyses and data exclusion

Group comparisons regarding sociodemographic, clinical/psychological and hair-related characteristics were conducted using *t*-tests for continuous variables and Fisher's exact tests for dichotomous variables. A multivariate analysis of variance (MANOVA) was further used to test for overall group differences in TICS subscales.

One GAD patient was excluded from hair cortisol analyses due to high outlying values which were probably associated with reported alcohol abuse and the use of antidepressant medication by that participant. With respect to the analysis of salivary cortisol, data exclusion was based on established criteria restricting analyses to those samples for which prespecified and objectively verified sampling times were not found to differ by more than 15 min for post-awakening samples (0 and 30 min; e.g. Dockray et al., 2008; Okun et al., 2010) and by more than 1 h for remaining diurnal samples (Kudielka et al., 2003). Since we were interested in examining total cortisol secretion over the day, rather than specific components of diurnal rhythmicity, these criteria were slightly relaxed to include cortisol data if the *mean difference* was within 15 min for post-awakening samples and within 1 h for the diurnal samples. Two-day mean values were calculated and used in subsequent analyses, if based on this criterion data

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