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The embarrassed brain : towards a neurobiology of generalized social anxiety disorder

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Elevated alpha-amylase but not cortisol in generalized social anxiety disorder

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Abstract

Stress system dysregulation is thought to increase the risk for anxiety disorders. Here we describe both hypothalamic pituitary adrenal (HPA) axis and autonomic nervous system (ANS) activity in basal non-challenging conditions and after 0.5 mg dexamethasone in generalized social anxiety disorder (gSAD) patients. To ensure stress-free sampling we collected saliva and determined cortisol and alpha-amylase (sAA), the latter a relative new marker of autonomic activity.

Forty-three untreated gSAD patients without comorbidity were compared with 43 age and gender matched controls in non-stressed conditions on sAA and cortisol after awakening, during the day (including late evening), and after a low dose (0.5 mg) of dexamethasone. Cortisol and sAA were analyzed with mixed models. Additional analyses were done with paired *t*-tests. Apart from the assessments in the morning, gSAD patients had significantly higher diurnal and post-dexamethasone 16.00h sAA levels. No differences between gSAD and controls in any cortisol measurements were found.

In conclusion, in gSAD in basal, non-stimulated conditions and after dexamethasone, we found hyperactivity of the ANS, as measured with sAA, but not of the HPA-axis. This suggests a relative increased activity of the ANS as compared to the HPA-axis, in line with the observed hyperarousal in gSAD.

Introduction

Social anxiety disorder (SAD; also known as social phobia) is one of the most common anxiety disorders (Stein, 2006). It is characterised by the fear of scrutiny by others. In specific SAD (sSAD) one or two situations are feared, e.g. speaking in public, while in generalized SAD (gSAD) most social situations are involved (American Psychiatric Association, 2000). The anxiety in gSAD is often accompanied by hyperarousal, such as increased heart rate, trembling, blushing and sweating. These clinical observations suggest an involvement of the stress system (De Kloet et al., 2005; Goldstein, 2003). However, whether the stress system is involved in gSAD is an unresolved question. Moreover, it is unclear under which conditions (basal versus challenge) and which parts of the stress system (autonomic nervous system (ANS) or the hypothalamic pituitary adrenal (HPA) axis) are involved. In gSAD, these systems have never been studied in concert, instead previous studies aimed to elucidate the role of these systems separately.

The function of the HPA-axis in SAD has been investigated by using cortisol levels as biological marker. In basal conditions no differences were found between gSAD patients and controls with respect to 24-hour urine cortisol levels (Potts et al., 1991; Uhde et al., 1994). Moreover, diurnal saliva cortisol levels of adolescent girls with SAD were comparable with those of healthy controls (Martel et al., 1999). The last study can be criticized since no distinction was made between subtypes of SAD. With some, but not all psychological challenges, HPA-axis dysfunction was reported. In one study gSAD patients had a significantly larger cortisol response to the Trier Social Stress Test than controls (Condren et al., 2002). Another study found a more outspoken dichotomous cortisol response after a stress challenge in gSAD compared to the control group: the cortisol responders in the group of gSAD patients had higher cortisol levels after the stress task than the cortisol responders in the control group, the cortisol non-responders in the gSAD group had lower cortisol levels than the non-responders in the control group (Furlan et al., 2001). However, in the study with adolescent girls mentioned above, Martel et al. (Martel et al., 1999) did not find any difference in cortisol responses between SAD and controls after a public speaking challenge. One study on the effects of a pharmacological challenge with dexamethasone in gSAD was published, reporting no differences between gSAD patients and controls in 24-hour urine cortisol levels (Uhde et al., 1994).

The function of the ANS has been studied in various ways. Stein and colleagues found higher noradrenaline levels in gSAD patients in one study, but could not replicate this finding in another (Stein et al., 1992, 1994). One study reported on higher baseline heart rate and blood pressure in gSAD patients compared to controls (Bouwer and Stein, 1998), whereas other studies did not (Grossman et al., 2001; Laederach-Hofmann et al., 2002; Gerlach et al., 2003).

A major drawback of autonomic measures, especially plasma (nor)adrenaline obtained by venipuncture, is the stress accompanying the sampling. Furthermore, heart rate and blood pressure are easily influenced by many factors, e.g. posture.

Recently, salivary alpha-amylase (sAA) has been proposed to reflect ANS activity (Chatterton, Jr. et al., 1996; Granger et al., 2007). In basal conditions in healthy volunteers, sAA

activity shows a diurnal profile with a decrease during the first 60 minutes after awakening and an increase during the rest of the day (Rohleder et al., 2004; Nater et al., 2007). sAA levels were relatively independent of several possible confounders like gender, body mass index (BMI), activity level, smoking, eating and drinking but significantly associated with chronic stress and stress reactivity in healthy volunteers (Nater et al., 2007).

Psychological and physiological challenges were followed by increases in sAA (Gilman et al., 1979; Bosch et al., 1996; Chatterton, Jr. et al., 1996; Chatterton, Jr et al., 1997; Rohleder et al., 2004; Rohleder et al., 2006; Nater et al., 2005; Nater et al., 2006; Kivlighan and Granger, 2006), although most studies failed to find significant correlations between adrenaline and noradrenaline, peripheral markers of the ANS, and sAA (Rohleder et al., 2004; Nater et al., 2006) indicating that stress-dependent sAA increases reflect changes of the ANS in general, not catecholamine increases. Furthermore, two pharmacological challenges provided direct evidence that sAA measures ANS activity. Propranolol (a non-selective beta-blocker), combined with a stressful task, resulted in lowering of sAA, showing the sensitivity of sAA to changes in adrenergic activity (Van Stegeren et al., 2006). Similarly, a yohimbine (an α_2 -receptor antagonist) challenge induced not only increases of peripheral noradrenaline levels but also increases of sAA levels compared to placebo. No correlations were found between adrenaline, noradrenaline and sAA, indicating that sAA reflects central noradrenaline release in stead of peripheral noradrenaline secretion (Ehlert et al., 2006). More research on sAA is discussed in the review of Granger et al. (2007).

The question remains whether there is a difference in basal autonomic activity in gSAD as compared to controls. Furthermore, we aimed to investigate the ANS and the HPA-axis in concert, since the specific balance between these stress systems, as was shown in experimental animal research, is often overlooked (Goldstein, 2003). Considering sAA as a measure of autonomic activity together with stress-free sampling of saliva stimulated us to use sAA to test basal autonomic activity in gSAD.

Here we report the awakening and diurnal rhythm of both cortisol and sAA, in basal conditions. In order to also measure the feedback sensitivity of the HPA-axis, we added a low-dose dexamethasone suppression test with 0.5 mg (Gaab et al., 2002), which is more sensitive to detect differences between different groups than higher dosages. We expected, based on former research, not to find differences in HPA-axis function in gSAD as compared to controls. However, we hypothesized that the ANS, as measured using sAA, would show hyperactivity in gSAD during the day.

Methods

Subjects

Forty-three patients with gSAD and 43 age (± 5 years) and gender matched healthy controls participated in this study. No life-time psychiatric comorbidity or clinically significant medical disorders, such as endocrinological disorders, were allowed. Patients were not currently treated for gSAD. Subjects were excluded in the case of alcohol consumption of over three units a day, using drugs of abuse or smoking more than 20 cigarettes a day. Use of psychotropic medication (including beta-

blocking agents) had to be stopped at least 14 days before the trial, with the exception of oxazepam (half life 4 to 15 hours) with a maximum dosage of 30 mg a day and only when used sporadically on an 'as needed' basis. It was allowed to be used up to 24 hours before the test days. No oral glucocorticoids were allowed in 6 months prior to the study. Because of the influence of estradiol on the HPA-axis, women were tested during the follicular phase of the menstrual cycle and women using oral contraceptives in the stop week. Women were asked about their menstruations in order to assess perimenopause (period of irregular menstruations before menopause) or menopause (last menstruation over a year ago). In case of any doubt, hormonal assessments were performed and discussed with a gynaecologist. Perimenopausal women were excluded, postmenopausal women were included.

Procedures

The protocol was conducted in accordance with the declaration of Helsinki and approved by the Medical Ethical Committee of the Leiden University Medical Center. Patients and controls were recruited by advertisements and interviews in local papers. Subjects contacted us and the investigator screened them by telephone on in- and exclusion criteria. If the criteria were met, the possible subjects were invited to visit our hospital. The possible controls were invited for a screening visit and received an information letter regarding the study by mail. In the case of suspected gSAD, patients were first invited for a diagnostic interview, then received the information letter and when eligible they were invited for the screening visit. Screening was performed within a 2-week period prior to the study. Before starting the screening, after complete description of the study, subjects signed the informed consent form. At the screening visit, DSM-IV diagnosis was confirmed and comorbidity was checked with the MINI Plus 5.0.0 (Van Vliet and De Beurs, 2007). Subjects were asked for their medical history and physical symptoms of illness. Then a physical examination was done, and on indication a laboratory investigation. The Liebowitz Social Anxiety Scale (LSAS) and the 17-points Hamilton Rating Scale for Depression (HRDS) were used in both groups to measure symptom severity (Liebowitz, 1987; Hamilton, 1960). Subjects with a HRDS score over 16 were excluded. A summary of the Structured Trauma Interview was used to make an inventory of experienced traumatic events (Draijer, 1989), because trauma might influence the HPA-axis even in people without PTSD.

In- and exclusion criteria were checked. After planning the two test days, salivettes and practical instructions were given.

The saliva samples were collected by the subjects at home during two non-working days. Patients and controls were specifically requested not to expose themselves to social situations or strenuous exercise during the test days, in order to measure stress markers in a basal, non-stressed condition. On test day I, samples were taken immediately after awakening, 30 minutes, 45 minutes and 60 minutes after awakening, at 1100 h, 1500 h, 1900 h, and 2200 h. At 2300 h they ingested a tablet of 0.5 mg dexamethasone. On test day II (the following day) subjects were asked to collect saliva samples at 900 h and 1600 h. After these test days subjects returned the salivettes through mail, and at arrival in our clinic the samples were frozen in -80 °C.

We measured the presence of dexamethasone in saliva in order to check on the reliability of the dexamethasone suppression test (DST) and to have an impression of the compliance of the subjects in both groups.

Instructions for saliva sampling

Saliva was obtained using salivette collecting devices, containing a cotton wad (Sarstedt, Rommelsdorf, Germany). For each sample subjects were asked to chew on this wad until the cotton was saturated with saliva, to put it back into the tube and then to keep it in the refrigerator. In order to improve compliance and to detect deviations from the protocol subjects were told: (1) to strictly follow the procedures and the time schedule for saliva sampling to obtain valuable data; (2) to record sample times and activities in the hour before sampling; (3) that we would be able to check compliance by looking at their cortisol curves. For the awakening sample, subjects were instructed to start saliva sampling immediately at awakening. Subjects were told to complete the early morning samples (awakening and 30 minutes, 45 minutes and 60 minutes afterwards) before brushing their teeth and having breakfast to avoid contamination of saliva with blood, food or drinks. They also were requested not to eat fruit or drink fruit juices and to thoroughly rinse their mouth with tap water before sampling saliva to avoid contamination.

Laboratory analysis of sAA and cortisol

The determination of cortisol in saliva was performed with a competitive electrochemiluminescence immunoassay ECLIA using a Modular Analytics E1 70 immunoassay analyzer from Roche Diagnostics (Mannheim, Germany). The sample volume was 20 μ L. The detection limit was 2 nmol/l (Van Aken et al., 2003).

The determination of sAA was performed with an enzymatic colorimetric assay using the maltoheptaoside (EPS) substrate on a P-module clinical chemistry analyzer (Roche, Mannheim, Germany) in 400-fold diluted saliva samples. The detection limit was 3 U/l (Lorentz, 1998).

The dexamethasone concentration was measured by a home-made radioimmunoassay (Department of Clinical Chemistry, Leiden University Medical Center, J. van Pelt). Dexamethasone was extracted from saliva and then analyzed by radioimmunoassay, which was performed with the antidexamethasone antibody from IgG Corporation (Nashville, Tennessee). The protocol from IgG Corporation was slightly modified. The lower limit of detection was 50 pmol/l (17.5 ng/l) and the reported cross-reactivity for cortisol is 0.04% (Weijtens et al., 1997).

Statistics

Outliers in cortisol levels were removed when they were above 50 nmol/l and higher than the mean + 3 S.D. Outliers in sAA levels were removed when they were above the mean + 3 S.D. The missing data were interpolated linearly. This was not possible for missing data on time points 1, 8, 9, or 10, because they were at the end or beginning of the day. Missing data on time point 1 and 8 were replaced by those of time point 2 and 7, respectively. Missing data on time point 9 and 10 the data were recorded as missing.

1. For evaluation of cortisol and sAA levels, Area Under the Curve with respect to ground (AUCg) values were first calculated for each subject separately and relative to the baseline value, for both the awakening cycle as well as for diurnal cortisol (11-22 h). We used the following formula for the calculation of the AUCg awakening : $((\text{value}_{t_1} + \text{value}_{t_2}) / 2) \times (t_2 - t_1) + ((\text{value}_{t_2} + \text{value}_{t_3}) / 2) \times (t_3 - t_2) + ((\text{value}_{t_3} + \text{value}_{t_4}) / 2) \times (t_4 - t_3)$; and the corresponding formula for the diurnal AUCg: $((\text{value}_{t_5} + \text{value}_{t_6}) / 2) \times (t_6 - t_5) + ((\text{value}_{t_6} + \text{value}_{t_7}) / 2) \times (t_7 - t_6) + ((\text{value}_{t_7} + \text{value}_{t_8}) / 2) \times (t_8 - t_7)$.

Subsequently, the thus summarized data were evaluated via mixed modeling, in order to take the paired nature of the data within matched pairs into account. The model is formulated as $Y_{ij} = \alpha_i + \beta X_{ij} + \gamma_1 CF_{1(ij)} + \dots + \gamma_k CF_{k(ij)} + \xi_{ij}$ where Y_{ij} are the modeled responses on the observed pairs, with $i=1, \dots, 43$ indicating the pair and $j=1, 2$ denoting the case and control status respectively of each of two observations within each i^{th} pair. We define $X_{ij}=1$ for $j=2$ and zero otherwise, such that β denotes the effect of substantive interest, contrasting cases from controls. The term $\gamma_1 CF_{1(ij)} + \dots + \gamma_k CF_{k(ij)}$ corrects for observed confounder effects, with CF_1, \dots, CF_k a set of k confounders. To obtain an analysis comparing cases with controls within pairs, we need to adjust for between-pairs heterogeneity, through assumption of the random effect $\alpha_i \approx \mathcal{N}(0, \sigma_\alpha^2)$. Conditional on both the between-pairs random effect and observed confounder adjustments, outcomes are assumed exchangeable with this model through assumption of residual normal error. We refer to Verbeke and Molenberghs (2001) for further details on the model.

For the cortisol data the mixed models were corrected for month of measurements (dark months: October through February; light months: March through September), time of awakening, and smoking (number of cigarettes a day), as was derived from yet unpublished data of our own center on determinants of salivary cortisol in 494 healthy controls. For the sAA data the mixed models were corrected for the use of coffee, smoking and the Body Mass Index as was based on the review of Granger et al. (2007). The evaluation of cortisol levels in the evening and sAA and cortisol levels subsequent to dexamethasone intake was based on separate analyses of the measurements taken at test day I at 2200 h and at test day II at 900 h and 1600 h, using the above described mixed model approach. For this statistical model, the assumption is that the residuals are normally distributed after the incorporation of the confounding factors in the model. Here, the residuals of both the sAA and the cortisol data were skewed. Therefore we performed a square root transformation for the sAA data, and for cortisol a log transformation, after which the residuals were investigated anew and found to be of normal distribution.

2. Separate analyses were implemented for patients and controls to investigate the presence of a cortisol awakening rise (CAR). Paired t -tests (two-sided) were carried out for cortisol measurements at 30 minutes post-awakening relative to the awakening measurements. These data were normally distributed.

3. All mixed model analyses were carried out through the SPSS 14.0 mixed model routine.

We are purposely presenting both the raw, unadjusted p -values as reported by the analyses and the Bonferroni corrected p -values.

Results

Subjects

Initially 46 patients and 54 controls were screened for this study. Three patients and eleven controls dropped out. For more detailed information on this see the flow chart in Figure 1. Forty-three patients with gSAD and 43 age (± 5 years) and gender matched healthy controls participated in this study. Subject characteristics are given in Table 1. The gSAD group was assessed within 13 months, the control subjects within 2.5 years.

Of the 43 patients and controls, some data were missing due to insufficient saliva collection. For all missing data, the matching data from the other group were removed from the analyses as well. Insufficient saliva was collected for cortisol measurement of test day II at 900 h in three subjects ($n=40$), and of cortisol test day II at 1600 h in one subject ($n=42$). Insufficient saliva was collected for sAA measurements of the AUC awakening in one subject ($n=42$) and at test day II 900 h in one subject ($n=42$). After the test days it appeared that one patient used a β -blocker for high blood pressure, but did not mention this before. The data of this patient were included in the analyses.

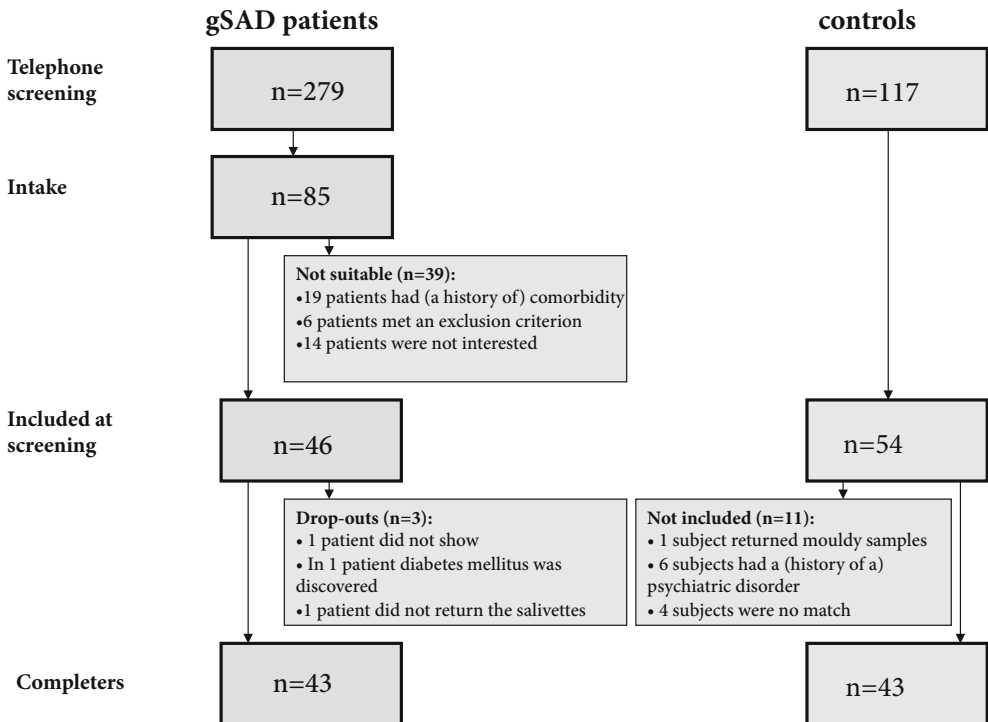


Figure 1. Flow chart of recruitment and inclusion process of gSAD patients and controls. The numbers of patients and controls that entered the various stages of the recruitment and inclusion process.

Table 1 Characteristics of gSAD patients and controls.

	gSAD patients (n=43)	Healthy controls (n=43)
CHARACTERISTICS		
Number of women / men	21 / 22	21 / 22
Age women / men (years)	42.1 (± 11.8) / 41.3 (± 13.9)	41.7 (± 12.2) / 41.4 (± 14.5)
Age total (years)	41.7 (± 12.8)	41.6 (± 13.3)
Smoking	16.3% (n=7)	14.0% (n=6)
Number cigarettes/day	8.6 (± 6.3)	10.3 (± 5.7)
Use of alcohol	86.0% (n=37)	74.4% (n=32)
Number of units alcohol/week	6.6 (± 4.2)	5.7 (± 3.7)
BMI	23.9 (± 3.4)	24.4 (± 3.9)
LSAS score	78.2 (± 19.6)	8.6 (± 5.6)
HRDS score	5.7 (± 3.1)	1.3 (± 1.5)
Trauma	49% (n=21)	40% (n=17)
MEDICATION		
Beta-blockers	2% (n=1)	0%
Oxazepam 10 mg (as needed)	2% (n=1)	2% (n=1)
Nasal glucocorticoids	2% (n=1)	0%
Inhaled glucocorticoids	2% (n=1)	2% (n=1)
Glucocorticoid crème	0%	5% (n=2)
Oral contraceptives	19% of women (n=4)	38% of women (n=8)

gSAD=generalized social anxiety disorder; BMI=body mass index; LSAS=Liebowitz Social Anxiety Scale; HRDS=Hamilton Rating Scale for Depression

Salivary cortisol

At test day I, we found no differences between gSAD patients and controls on the AUC cortisol after awakening as analyzed with mixed models and corrected for the confounders ($p=0.864$) (see Figure 2 and Table 2). In each group we compared cortisol levels at 30 minutes after awakening with cortisol levels directly at awakening with a paired sample t-test. In both the gSAD and control group no significant differences were found between these time points (mean = -2.62, S.D. = 9.23, $p = 0.07$; mean = -0.035, S.D. = 5.56, $p = 0.967$), reflecting the absence of a CAR in both groups (see Figure 2).

Mixed models analysis with correction for confounders also showed no differences between both groups in the AUC diurnal cortisol and the cortisol level at 2200 h ($p=0.773$; $p=0.201$) (see Figure 2 and Table 2).

Compliance was checked in both groups. Dexamethasone was present in the saliva at day II of all subjects, except for three controls and one patient. Since an obvious suppression of their cortisol levels was found, they probably did ingest the dexamethasone. At test day II post-dexamethasone cortisol levels (900 h and 1600 h) did not differentiate between the groups ($p=0.920$; $p=0.256$) (see Table 2 and Figure 2).

Taken together, we did not observe significant differences in saliva cortisol at any measure of the HPA-axis we used.

Salivary alpha-amylase

In contrast, sAA did show statistically significant differences between patients and controls as analyzed with mixed models, after correction for the confounding factors. On test day I in the morning, during the first hour after awakening, we did not find significant differences in the AUC sAA awakening ($p=0.114$) (see Figure 3 and Table 2). However, during the day (from 1100 h to 2200 h), the AUC sAA diurnal was much higher in gSAD patients as compared to controls ($p=0.022$). At 1500 h sAA levels were almost twice as high in patients as compared to controls. For details see Figure 3 and Table 2.

On test day II, post-dexamethasone sAA levels were significantly higher in gSAD patients compared to controls at 1600 h ($p=0.02$) (see Table 2, and Figure 3), but not at 900 h ($p=0.059$) (see Table 2 and Figure 3). In Table 2 also the Bonferroni corrected p -values are given.

Table 2 Cortisol (log transformed) and sAA (sqrt transformed) data in gSAD patients and controls as analyzed with mixed models (within matched pairs)

		Mean gSAD	Co	<i>F</i>	<i>p</i>	<i>p</i> (bonf. corr.)	<i>n</i>
Cortisol (nmol/l)							
Day I	AUC awakening	962.2	1060.9	0.030	0.864	-	43
	AUC diurnal	4993.6	5486.5	0.084	0.773	-	43
	Late evening (2200 h)	3.5	4.5	1.687	0.201	-	43
Day II	Post-dex. 900 h	4.1	4.5	0.010	0.920	-	40
	Post-dex. 1600 h	3.384	3.029	1.307	0.256	-	42
Amylase (U/ml)							
Day I	AUC awakening	9299.145	6959.123	2.612	0.114	-	42
	AUC diurnal	225147.69	146808.58	5.413	0.022*	0.044*	43
Day II	Post-dex. 900 h	191.776	130.758	3.794	0.059	-	42
	Post-dex. 1600 h	327.542	206.047	5.628	0.020*	0.040*	43

sAA=salivary alpha-amylase; gSAD=generalized social anxiety disorder; Co=controls; AUC awakening = the area under the curve of cortisol levels at awakening, 30 minutes, 45 minutes and 60 minutes after awakening; AUC diurnal= the area under the curve of cortisol levels at 1100 h, 1500 h, 1900 h and 2200 h. Late evening cortisol is the cortisol level at 2200 h. Post-dex. are the data following the ingestion of 0.5 mg dexamethasone. These data reflect mixed models analysis of the cortisol and sAA data, corrected for confounders. The cortisol data were log-transformed. For the sAA data a square root transformation was performed. The *F* and *p*-values reflect analyses with the transformed data. *P* (bonf. corr.) reflects the bonferroni corrected significant *p*-values. * means that the data were statistically significant. For cortisol the confounders that were taken into account were month of measurements, time of awakening, and smoking, for sAA the confounders that were taken into account were use of coffee, smoking, and the Body Mass Index.

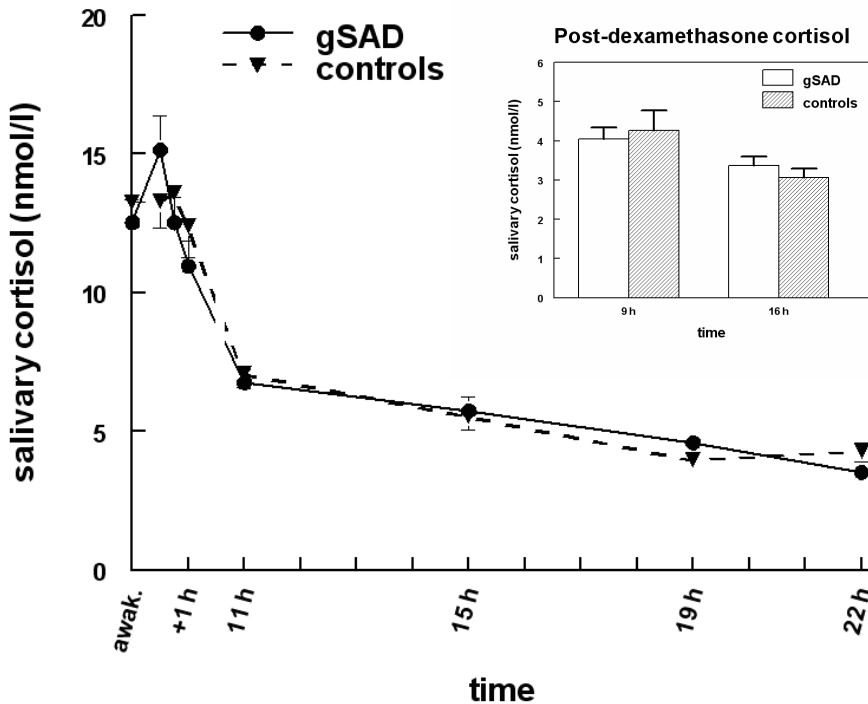


Figure 2. Awakening, diurnal and post-dexamethasone salivary cortisol levels (\pm S.E.M.) in gSAD patients and controls. Awak. = awakening samples, they were taken at the moment of awakening, and after 30 minutes, 45 minutes and 1 hour after awakening at test day I. The diurnal samples were taken at 1100 h, 1500 h, 1900 h and 2200 h at test day I. Post-dexamethasone salivary cortisol levels: 900 h and 1600 h are the time points at test day II, the day following the ingestion of dexamethasone.

Discussion

Here we show that gSAD patients differ from healthy controls in their diurnal sAA and post-dexamethasone sAA level at 1600 h, but not in their cortisol levels. This suggests an imbalance between the two branches of the stress system. Several arguments indicate that the increased diurnal sAA levels really indicate higher ANS activity in gSAD. However, as discussed in the introduction, previous studies in gSAD with traditional markers of ANS activity like (nor)adrenaline, heart rate and blood pressure were contradictory. First this could be explained by the findings that sAA as a marker for the ANS is relatively independent from several possible confounding factors as was reported before (Nater et al., 2007), while (nor)adrenaline levels, heart rate and blood pressure, are easily influenced by many factors, including posture and venipuncture. Second, we assessed sAA several times a day contrary to the single or closely clustered assessments of the 'classical' parameters.

The diurnal sAA levels and patterns we describe here were very similar to the sAA curves that were found before (Rohleder et al., 2004; Nater et al., 2007), indicating the validity of our results. Although it was not the aim of our study, we found no effects of dexamethasone on sAA. This means that the post-dexamethasone ANS hyperactivity we found in gSAD probably also reflects basal hyperactivity as we found on test day I.

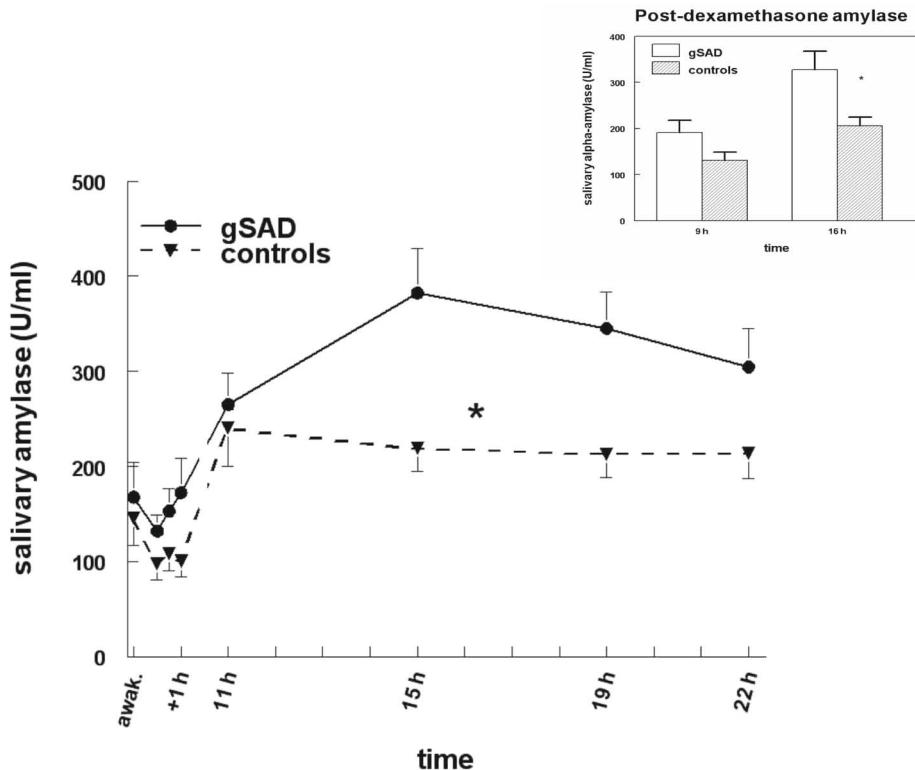


Figure 3. Awakening, diurnal and post-dexamethasone salivary alpha-amylase levels (\pm S.E.M.) in gSAD patients and controls. Awak. = awakening samples, they were taken at the moment of awakening, and after 30 minutes, 45 minutes and 1 hour after awakening at test day I. The diurnal samples were taken at 1100 h, 1500 h, 1900 h and 2200 h at test day I. Post-dexamethasone salivary alpha-amylase levels: 900 h and 1600 h are the time points at test day II, the day following the ingestion of dexamethasone.

* means a statistical difference between patients and controls in sAA levels as analyzed with mixed models ($p=0.009$).

With respect to cortisol levels we did not find differences between gSAD patients and healthy controls, as was previously shown by others (Potts et al., 1991; Uhde et al., 1994; Martel et al., 1999).

In contrast to what might be expected, neither in the gSAD patients nor in the controls a cortisol awakening rise (CAR) was found. This may be the consequence of asking the participants to sample saliva cortisol during rest days. It has been reported that the CAR is smaller in weekend days as opposed to working days (Kunz-Ebrecht et al., 2004). It also might be the effect of non-adherence to the protocol (Thorn et al., 2006), the effect of the time of awakening, which apparently is different in weekend days (Clow et al., 2004), or the effect of the great variation of cortisol levels in the general population.

No statistically significant differences between the groups were observed in the DST, with both groups showing suppression of cortisol. HPA-axis suppression in the gSAD group was comparable to those in healthy controls being in line with a previous report (Uhde et al., 1994). Thus it could be concluded from the present study that in basal conditions the HPA-axis function is

normal, whereas there is an increased activity of the autonomic system itself.

We were the first to investigate the HPA-axis and the ANS together in basal non-stimulated conditions in gSAD, and had the opportunity to directly compare these two systems in the same group of patients and controls. Other strengths of the study are that we used a relatively large group of patients with individually matched controls. The patients were suffering from pure gSAD, no lifetime comorbidity was allowed. For cortisol we tried to prevent the influence of confounding factors in several ways. Patients and controls were matched for age and gender. Subjects that were heavy smokers or drinkers were excluded. Furthermore, we corrected for several remaining confounding factors in the statistical analyses. For sAA we also took several possible confounders into account, based on the review of Granger et al. (Granger et al., 2007). Another strength is that cortisol and sAA were measured in saliva (Gozansky et al., 2005; Rohleder et al., 2006), which has the advantage of non-stressful sampling as opposed to measurements in blood. In addition, saliva samples were collected at home, on non-working and non-stressful days, without engaging in social situations. This approach secures equal conditions in both groups, without confounding factors as anticipatory and social anxiety in the gSAD group.

In asking participants to sample saliva at home we optimized the chances that assessments took place under basal conditions. In doing so we followed many other studies (Clow et al., 2004). However, unsupervised sampling at home may decrease compliance. We took several measures to control for this possibility. First, we stimulated compliance by asking the participants to write down the time of sampling and the activities they did the hour before sampling, and by informing them about our plans to check compliance, as it has been demonstrated that such a procedure enhances compliance (Kudielka et al., 2003). Second, we checked saliva dexamethasone levels and found no indications of non-compliance in any of the participants. Nevertheless, we cannot rule out that non-compliance to the protocol influenced our data on cortisol as well as sAA. Hence, we might have missed existing differences between gSAD patients and controls at the cortisol and sAA awakening responses. Furthermore, our data might have been of more value if we had measured full awakening responses of both markers after the DST.

In conclusion, under basal conditions we found no changes in HPA-axis activity but, in contrast, an increased autonomic activity. This suggests an imbalance in the stress-system probably by relative increased activity of the autonomic system as compared to the HPA-axis. These findings are in line with the hyperarousal observed in gSAD patients, as it might perhaps be the result of the hyperactivity of the ANS. Alternatively, the hyperactivity of the ANS might be the result of the chronic stress accompanying the continuing fear to be humiliated by others. Further research could be directed at the associations between hyperarousal and sAA in gSAD and at differences in ANS activity between gSAD and other anxiety disorders.

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