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Familial influences on basal salivary cortisol in an adult population

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Summary To understand the underlying genetic and environmental sources of individual variation in basal cortisol levels, we collected salivary cortisol at awakening and at six fixed time points during the day in adult twins and their singleton siblings. Reported time of awakening was verified with heart rate and body movement recordings. Cortisol data were available for 199 MZ twins, 272 DZ twins and 229 singleton siblings from 309 twin families. No differences in cortisol means and variances were found between twins and singleton siblings. Additionally, the correlations for DZ twins and siblings were not significantly different, indicating generalizability of twin study results to the general population. Genetic model fitting showed heritability for cortisol levels during the awakening period (34% for cortisol level at awakening and 32% for cortisol level at 30 min after awakening) but not for cortisol levels later during the day. The current study shows that, while cortisol levels in the awakening period are influenced by genetic factors, cortisol levels throughout most of the day are not heritable, indicating that future gene finding studies for basal cortisol should focus on the first hour post-awakening.
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1. Introduction

Cortisol is an important steroid hormone in the regulation of normal physiology. It is the end product of the hypothalamus–pituitary-adrenal (HPA) axis. In response to disturbance of homeostasis due to physical or psychological influences, corticotrophin releasing factor (CRF) is expressed in the paraventricular nucleus of the hypothalamus and acts to stimulate the secretion of adrenocorticotrophic hormone (ACTH) in the pituitary. ACTH travels to the adrenals, where it stimulates the production of cortisol in the outer layer of the adrenal cortex. By a negative feedback mechanism, cortisol inhibits the production of both ACTH and CRF, thereby inhibiting its own secretion. Under influence of the central nervous system about 10–15 well-defined ACTH driven pulses of cortisol are secreted over 24 h, resulting in cortisol's well-known circadian rhythm that is characterized by

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peak levels in the early morning and a nadir around midnight. Normally, stress-induced secretion is superimposed on the basal circadian rhythm. When the HPA axis is deregulated, however, for example by continued or frequently repeated stress challenges, basal cortisol may be chronically secreted in excess, with potentially harmful effects. Prolonged glucocorticoid exposure may lead to muscle atrophy, decreased sensitivity to insulin, hyperlipidemia, hypercholesterolemia, impairment of growth (Meaney et al., 1991), osteoporosis (Adachi, 2001), immune destabilization (Bateman et al., 1989), hypertension and cardiovascular disease (Mantero and Boscaro, 1992; Girod and Brotman, 2004). In addition, deregulated HPA axis activity is a predictor for diabetes and stroke (Rosmond and Björntorp, 2000).

Large individual differences exist in the diurnal levels of cortisol (Smyth et al., 1997) and a possible source of this variation is genetic makeup. Several twin studies have been conducted to determine the influence of genetic and environmental factors on basal cortisol (for a review see Bartels et al., 2003b). The majority has focused on one basal cortisol sample during the morning hours (07:45–09:00 h, not related to awakening), and only two studies in adults (Linkowski et al., 1993; Wüst et al., 2000a) and one study in children (Bartels et al., 2003a) report on the heritability of basal cortisol collected during an entire day. Linkowski and coworkers (1993) determined cortisol in blood samples taken every 15 min for 24 h in 21 twin pairs. Genes influenced the timing of the nocturnal nadir and the proportion of overall temporal variability associated with pulsatility. No genetic effects were detected for the 24-h mean and the timing of the morning acrophase. Wüst et al. (2000a) collected eight saliva samples from awakening until 20:00 h in 104 twin pairs and reported significant genetic control (40 and 48%) for the different measures of the early morning acrophase, while cortisol variation during the rest of the day was predominantly under shared and non-shared environmental control (Wüst et al., 2000a). The study by Bartels and colleagues (2003a) in 216 children aged 12 showed a similar genetic pattern as the adult twin study by Wüst et al. (2000a) with significant genetic influences on cortisol levels an hour post-awakening (about 57%), and only environmental influences for the afternoon and evening levels of cortisol.

These studies indicate that genes influence the cortisol levels in the early morning, no matter what age, but not during the rest of the day. The present study increased the power to detect influences of genetic and common environmental factors by increasing the sample size and by adding the singleton siblings of the twins to the design (Posthuma and Boomsma, 2000). This extended twin design has the additional advantage that it allows testing of the assumption that twin results can be generalized to singletons.

2. Methods

2.1. Subjects

Subjects were registered with the Netherlands Twin Register (NTR) and were originally selected for a genetic linkage study for anxious depression (Boomsma et al., 2000; Middeldorp et al., in press). Briefly, families were selected when two siblings (dizygotic twin pair, sib-twin pair, or sib-sib pair) were extremely discordant or concordant for anxious depression. In addition to the sibling pair, all registered family members were recruited for the study. The resulting distribution of anxiety, neuroticism and depression scores was near-normal with only mild kurtosis. Of the first 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for a cardiovascular and hormonal ambulatory monitoring study. Of these, 192 refused participation or were excluded. Exclusion criteria were pregnancy, heart transplantation, pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. As the collection of saliva was added to the study protocol after the study started, a further 98 subjects did not participate, leaving a total of 718 subjects who took part in the cortisol collection. For 18 subjects the cortisol data had to be discarded because they used corticosteroid medication (12 subjects), or had unreliable profiles (six subjects) due to working a night shift or uncertainty about the sample order. The final study sample consisted of 700 subjects, including 199 MZ twins (75 males), 272 DZ twins (94 males) and 229 singleton siblings (88 males), who were tested in two data collection waves. In total, 309 families participated. Both twins participated in 192 families, and in 112 of these families one or more additional siblings were present. In 54 families, data were available for one twin individual and one or more singleton siblings for 54 families. In 13 families, data were only available for two or more singleton siblings, while in 50 families data were present for one individual (twin or sib) only.

Zygosity of the twins was determined by DNA typing. The Ethics Committee of the Vrije

Universiteit approved of the study protocol and all procedures were carried out with adequate understanding and written consent of the subjects.

2.2. General procedure

Subjects were requested to refrain from intense physical exercise on both the preceding and the ambulatory monitoring days. Subjects were visited at home early in the morning, before starting their normal activities. They were subjected to an interview on health status and received instructions on the saliva sampling for cortisol assessment. In addition, a 24-h electrocardiogram (ECG) and impedance cardiogram (ICG) recording was made using the Vrije Universiteit-Ambulatory Monitoring System (VU-AMS) ambulatory monitor (De Geus et al., 1995; De Geus and van Doornen, 1996) that includes a vertical accelerometer. Furthermore, every 30 min blood pressure was recorded using a Spacelabs 90207 ambulatory blood pressure monitor (Redmont, Washington, USA). Results on cardiovascular measures have been published elsewhere (Kupper et al., 2004, 2005). Subjects wore the VU-AMS monitor the entire day and night until after awakening the next morning. In a chronological diary, subjects recorded the actual times saliva collection took place, and indicated any deviations from the instructions.

2.3. Saliva collection

Saliva sampling was performed using Salivette® sampling devices (Sarstedt, Rommelsdorf, Germany). Subjects were instructed to chew gently on the polyester swab for 45 s to obtain the desired amount of saliva. They were asked to refrain from brushing their teeth and consuming food and drinks from half an hour before saliva sampling. The first sample was collected in the presence of the researcher, at the start of the measurement day. Instructions were to take the next samples at 11:00, 15:00, 20:00, 22:30 h (or prior to going to bed, when earlier), upon awakening the next morning (preferably while still in bed), and 30 min post-awakening. This last sample was only available for the 428 subjects who participated in a second data collection wave.

2.4. Cortisol analysis

All samples were stored frozen at the laboratory at a temperature of $-25\,^{\circ}C$. Cortisol concentration was determined in Düsseldorf, Germany, in two batches. In the first batch, consisting of the samples

of 272 subjects from 166 families, cortisol concentration was determined by a time-resolved immunoassay with fluorescence detection (DELFIA, see Dressendorfer et al., 1992). Intra and inter assay variability of this method were less than 10 and 12%, respectively. In the samples of the 428 subjects (from 143 families) of the second batch, cortisol concentration was determined using a commercial competitive chemiluminiscence immunoassay (LIA, IBL Hamburg, www.ibl-hamburg.com). Intra and inter assay variability of this method were less than 7.7 and 11.5%, respectively.

2.5. Measures and outlier detection

In addition to the seven diurnal cortisol samples, we computed the cortisol awakening response (CAR) by subtracting the cortisol concentration at awakening from the cortisol concentration 30 min later. When cortisol concentration reached values more than 3 times the standard deviation above or below the mean for that sampling time, the sample was discarded (this happened in 1% of all samples).

2.6. Statistical analysis

2.6.1. Confounders
In the past decades, a multitude of research has been published on factors influencing cortisol. The main factors implicated are age, gender, smoking, mood, bodily composition, contraceptive pills, sleep duration, sleep quality, and awakening time, although many studies report contradictory results (Deuschle et al., 1997; Knutsson et al., 1997; Wüst et al., 2000b; Ukkola et al., 2001). Therefore, we decided to test all of these potential confounders for their influence on basal diurnal cortisol using regression analyses in SPSS (SPSS, Inc., Chicago, USA). The effects of sex, age, current mood state, (as measured by the POMS (Wald and Mellenbergh, 1990)), body mass index (BMI), sleep quality (assessed by the Groningen Sleep Quality Scale, Meijman et al., 1988), reported sleep duration, current habitual smoking status (yes/no), and oral contraceptives use on the cortisol samples were tested. Since two methods (DELFIA and LIA) were used to determine the cortisol concentration in saliva, we also treated the type of immunoassay as a possible confounder in our genetic analyses.

2.6.2. Genetic modeling
To answer the question to which extent genes, common environment and non-shared environment contribute to the variance of basal cortisol, a biometrical genetic model was fitted to the

observed data using the structural equation modeling program Mx (Neale et al., 2003). A series of unconstrained models was fitted to test the assumptions of the extended twin model. In this series, we first tested the equality of means and variances for MZ twins, DZ twins, and singleton siblings and examined the presence of sex effects on the means and variances. Then we tested whether cortisol determination method significantly affected the means. Finally, we tested for heterogeneity of correlations of males versus females and of DZ twins versus singletons. The resulting most parsimonious saturated model indicated to which extent we could limit the specification of the genetic models and provided correlations for the MZ group and the DZ/sibling group.

In a twin study, the observed variance can be decomposed in four possible latent sources of variance. The two environmental sources are environmental effects that are shared by members of a family (C), and environmental effects that are unique to each member of a family (E). Two kinds of genetic effects are distinguished: additive genetic effects (A) and non-additive genetic effects. Non-additive genetic effects include dominance effects (D) and epistasis. Dominance describes the interaction between alleles at the same locus (Neale and Cardon, 1992). In a design that includes identical twins, fraternal twins and sibling pairs, estimates of C and D are confounded, and the observed variances and covariances only provide sufficient information to model either an ACE model or an ADE model, but not both. Based on the pattern of twin and sibling correlations we choose which model was more appropriate. For MZ, DZ twins and sibling pairs alike, similarity in shared environmental influences was fixed at 100%. Similarity of additive genetic influences was fixed at 50% for siblings and DZ twins and at 100% for MZ twins. In the case of dominance (when the MZ correlation is more than twice the DZ correlation), similarity of dominant genetic influences was fixed at 25% for siblings and DZ twins and at 100% for MZ twins. Per definition, there is no similarity in the non-shared environmental influences for all three types of sibling pairings. For each of the cortisol samples, a full univariate ACE or ADE model (Neale and Cardon, 1992) was tested against the nested more parsimonious AE, CE or E models. The resulting best fitting model indicated how much of the variance is attributed to genetic influences and how much is attributed to environmental influences. Throughout, nested models were compared using the likelihood ratio test. To determine whether shared genetic influences would underlie the two cortisol levels of the

awakening period, a bivariate full ADE model in Cholesky decomposition was tested against more parsimonious models (AE and E models). The Cholesky decomposition imposes a structure of stratification in several shared latent factors. In the case of our bivariate analysis, there is a main factor that loads on both variables, followed by a second factor that loads on the last variable only. In the full model all variance components (A, D and E) are structured this way. Significance of the individual path coefficients was tested by constraining paths to zero and comparing the fit with likelihood ratio tests. In the bivariate model, the heritability of the CAR was also estimated. Because of the design of the model, CAR heritability reflects the remaining genetic influence on the difference in cortisol levels between the two samples, after removing the heritability for the two individual mean cortisol levels. Akaike's Information Criterion ($AIC = \chi^2 - 2df$, Akaike, 1987) was calculated for each of the univariate and bivariate models. AIC offers a quick approach to judging the fit of nested models and models that are not nested, like an AE and CE model. Those with lower (i.e. larger negative) values fit better than models with higher values.

3. Results

3.1. Descriptive statistics

Of the 700 subjects that participated in the saliva collection for cortisol determination, 587 had a complete diurnal profile of six samples (of these, 376 subjects participated in the second data collection wave, and provided seven samples). For 10 subjects less than four samples were present. Subjects complied moderately well with the instructed sampling times. Between 74 and 85% of the time-bound samples (11:00, 15:00, 20:00 and 22:30 h) were reported to be taken within 15 min of the requested sampling time. Of the samples outside the 30 min window, the majority was taken later than the requested sampling time (1–3% was taken more than 15 min earlier, 21–25% was taken more than 15 min later, and 14–17% was taken more than 30 min later than the required sampling time).

Looking at the individual profiles, most subjects (89%) showed the well-known diurnal rhythm of cortisol. For 77 subjects for whom both early morning samples were present, the cortisol awakening response was negative. Fig. 1 shows the diurnal cortisol profile for those with a normal
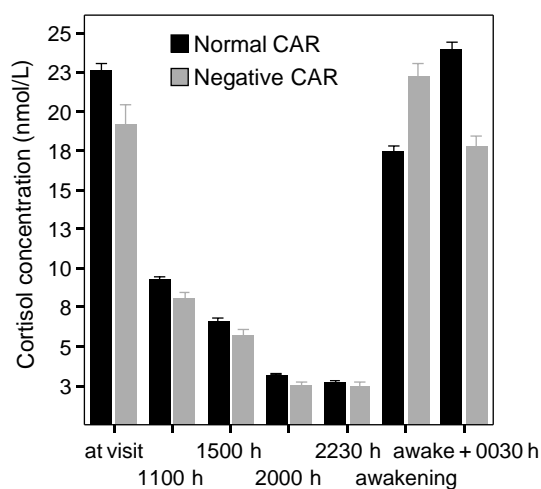
Figure 1 Cortisol diurnal profile for normal and negative awakening response groups. Represented cortisol means are corrected for method of cortisol determination. Error bars represent the standard error. CAR, cortisol awakening response.

awakening response (black bars) and those with a negative response (gray bars). It is possible that these subjects showing a negative response really do have a deviant response to awakening. However, an alternative explanation might be that subjects woke up earlier than they reported, and that their data therefore represent the down stroke of the morning acrophase. To test this alternative explanation, we exploited the fact that we had simul-taneous recordings of heart rate and body movement on the sampling days. For 59 of the 77 subjects an ECG/motility recording of the early morning hours was available. For 18 subjects ECG/motility data were missing due to signal loss in the middle of the night. We identified an earlier awakening moment than reported in 80% of the subjects with available ECG/motility data. Fig. 2 shows the combined ECG/motility signal for one subject with a discrepancy between his actual awakening time and his reported awakening/sampling time. The time difference between actual awakening and reported awakening was 42 min (range = 10 min–02:15 h), which suggests that the negative awakening response is an artifact caused by sampling after the actual awakening response occurred. To determine whether these results on earlier awakening are also found in the group with a normal awakening response, a random sample of 77 subjects was drawn from those with normal awakening responses. For eight of these randomly drawn subjects ECG/motility data were missing due to signal loss in the middle of the night. In 87% of the subjects with available ECG/motility data the reported awakening time corresponded with the actual awakening as judged from the ECG and body movement recordings. These observations indicate that waking up earlier than reported was indeed responsible for the majority of the apparent negative cortisol awakening responses. Because the awakening response was assessed incorrectly
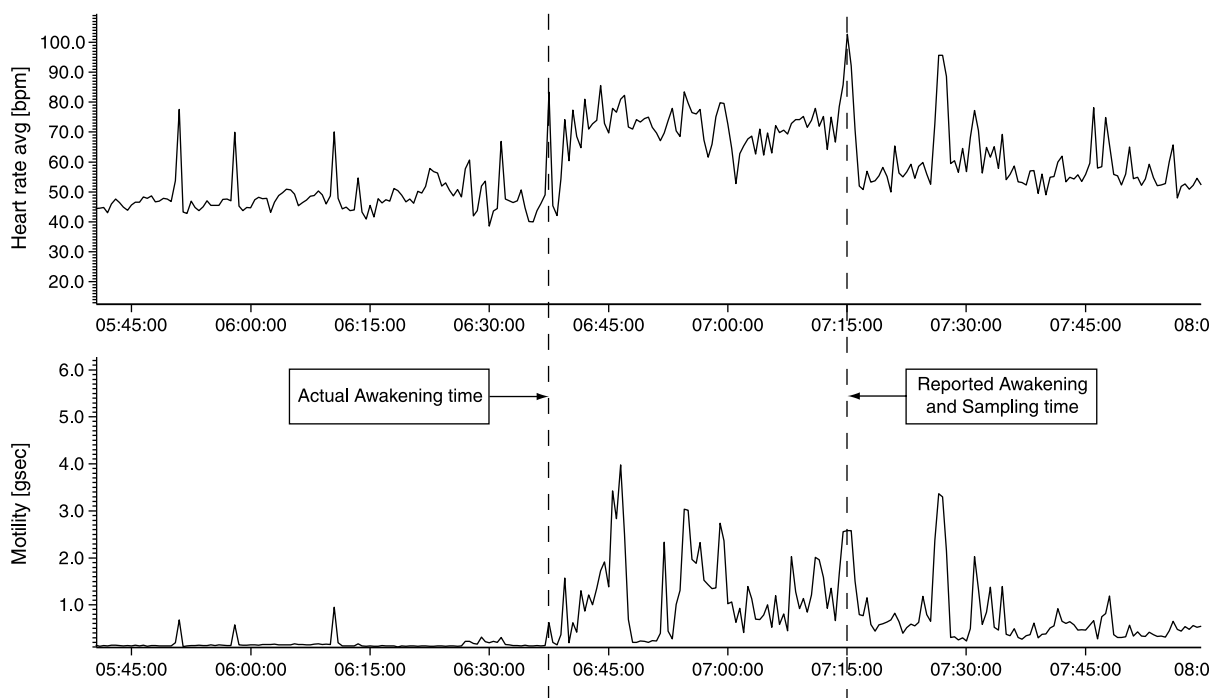

Figure 2 Example of an ECG/motility signal during the awakening period of a subject showing a negative CAR.

Table 1 Descriptive statistics and twin correlations for diurnal cortisol.

Sample	N	Mean sampling time (sd)	Mean cortisol (sd) in nmol/L	MZ correlation	DZ/sib correlation
At visit	667	8:35 h (01:07 h)	20.87 (10.49)	.33 (.13-.49)	.16 (.05-.26)
11:00 h	655	11:11 h (00:30 h)	8.69 (4.07)	.11 (.00-.34)	.09 (.00-.19)
15:00 h	675	15:18 h (00:42 h)	6.14 (3.07)	.08 (.00-.28)	.13 (.03-.23)
20:00 h	638	20:12 h (00:31 h)	2.85 (1.60)	.20 (.00-.42)	.18 (.08-.29)
22:30 h	649	22:39 h (00:28 h)	2.37 (1.95)	.00 (.00-.18)	.19 (.09-.29)
Awakening	563	7:16 h (00:51 h)	15.68 (7.36)	*.52 (.26-.68)*	*.12 (.00-.24)*
Awake + 00:30 h	319	7:45 h (00:58 h)	23.91 (8.23)	*.41 (.00-.67)*	*.14 (.00-.30)*

N, number of subjects; sd, standard deviation; CAR, cortisol awakening response. Means were corrected for cortisol determination method. For the correlations the 95% confidence intervals are given in parentheses. Italic: variables for which an ADE instead of an ACE model is fitted.

in these subjects showing a negative CAR, their awakening response samples were excluded from further analyses.

Table 1 shows the descriptive statistics for the cortisol samples. A clear circadian rhythm can be observed. The average cortisol concentrations at the seven sampling times were significantly different from each other and moderately correlated (between .08 and .47), with the exception of the awakening and 30 min post-awakening samples which were highly correlated ($r = .65$, $p = .000$).

3.2. Confounders

When examining the association between the cortisol concentration at the time points and the potential confounding variables (sex, age, current mood state, BMI, sleep quality, sleep duration, current habitual smoking status (yes/no), and oral contraceptives use), no association reached the .01 significance level.

3.3. Genetic model fitting

Model fitting showed that the means and variances for MZ twins, DZ twins, and singleton siblings could be constrained to be equal. Male correlations were equal to female correlations and correlations were not significantly different between DZ twins and singletons. These results indicate that DZ twins and siblings may be treated as individuals from one group in further analyses. There was a significant mean effect of the method used to determine the cortisol concentration. Mean cortisol levels were higher in the second group of participants (when LIA was used) than in the first group of participants (when DELFIA was used). We therefore kept type of immunoassay as a covariate on the mean and the variance in the variance decomposition models.

Estimation of the effect of type of immunoassay on the variance was performed following Purcell's gene-interaction model (Purcell, 2002).

The twin correlations from the final, most parsimonious model are presented in the final two columns of Table 1. There were minimal differences in MZ and DZ/sib correlations during most of the day. Based on the pattern of correlations, substantial familiar influences are only present for the visit sample and the early morning measures of cortisol. In case of the two early morning measures (awakening, 30 min post-awakening), MZ correlations were more than twice as high as the DZ/sib correlations, suggesting the presence of dominance genetic effects. Therefore, we fitted ADE models for these measures. We continued model fitting with the most parsimonious saturated model, i.e. a model with equal means, variances and two correlations, including type of immunoassay as a covariate. Table 2 summarizes the model fitting results for all univariate variance decomposition models. The accompanying univariate model estimates and 95% confidence intervals are shown in Table 3.

3.3.1. Univariate genetic analyses
For both the awakening sample and the 30 min post-awakening sample, dismissing the dominance genetic effect did not cause a significant worsening of fit. In the AE model, additive genetic factors accounted for 33% of the variance in cortisol levels at awakening, while non-shared environmental influences accounted for the remaining 67% of the variance. For the cortisol concentration at 30 min post-awakening, genetic factors explained 34% of the variance, while non-shared environmental factors explained 66% of the variance.

For the daytime samples at 11:00 and 15:00 h, leaving out both shared environmental and genetic influences from the model (E model) did not cause a

Table 2 Summary of the univariate model fitting results.

Model	−2LL	df	Δχ²ᵃ	Δdf	p-value	AIC
At visit						
ACE	2029.880	661				
AE	**2029.880**	**662**	**0.000**	**1**	**1.000**	**−2.000**
CE	**2032.287**	**662**	**2.407**	**1**	**0.121**	**0.407**
E	2045.544	663	15.664	2	0.000	11.664
11:00 h						
ACE	1299.591	649				
AE	1299.817	650	0.226	1	0.635	−1.774
CE	1299.591	650	0.000	1	1.000	−2.000
E	**1302.269**	**651**	**2.678**	**2**	**0.262**	**−1.322**
15:00 h						
ACE	1131.653	669				
AE	1132.588	670	0.935	1	0.334	−1.065
CE	1131.653	670	0.000	1	1.000	−2.000
E	**1135.770**	**671**	**4.117**	**2**	**0.128**	**0.117**
20:00 h						
ACE	754.749	632				
AE	**755.23**	**633**	**0.481**	**1**	**0.488**	**−1.519**
CE	**754.765**	**633**	**0.016**	**1**	**0.899**	**−1.984**
E	764.198	634	9.449	2	0.009	5.449
22:30 h						
ACE	3906.054	641				
AE	**3907.763**	**642**	**1.709**	**1**	**0.191**	**−0.291**
CE	**3906.054**	**642**	**0.000**	**1**	**1.000**	**−2.000**
E	3911.967	643	5.913	2	0.052	1.913
Awakening						
ADE	1451.323	557				
AE	**1451.323**	**558**	**0.000**	**1**	**1.000**	**−2.000**
E	1460.687	559	9.364	2	0.002	5.364
Awake+00:30 h						
ADE	814.871	314				
AE	**815.609**	**315**	**0.738**	**1**	**0.390**	**−1.262**
E	820.233	316	5.362	2	0.032	1.362

−2LL, twice the negative log likelihood; df, degrees of freedom; AIC, Akaike's Information criterion. When the increase in χ^2 (= $\Delta\chi^2$) is not significant ($p > .05$), the most restrictive model is accepted. **Bold** indicate(s) the most parsimonious model(s). For the visit, 20:00 and 22:30 h sample we could not distinguish between the AE and CE model, both provided a better fit than the ACE model.

ᵃ Fitted against less restrictive model.

significant increase in χ^2. This means that the cortisol concentration at these time points is completely determined by non-shared environmental factors. The 20:00 and the 22:30 h sample showed a significant worsening of fit when both genetic and common environmental factors were left out of the model, indicating that there is an influence of familial factors on cortisol levels at these time points. Statistical power, however, was insufficient to discriminate between genetic influences and shared environmental influences, since the estimates for A and C were quite small (<22%).

For the first sample, taken during the visit of the researcher, the pattern of twin correlations and the estimates in the full ACE model indicate that the AE model is the most likely model, although statistical power is insufficient to discriminate between genetic and shared environmental factors. In the AE model, variance in cortisol concentration is for 29% explained by genetic influences.

3.3.2. Bivariate genetic analysis

To determine whether the same genes underlie the individual differences in the two cortisol samples of the awakening period (awakening and 30 min post-awakening), they were analyzed in a bivariate analysis, for which the initial ADE model is illustrated in Fig. 3. The dominance genetic factor could be dismissed from the bivariate model without a significant loss of fit, thereby reducing it

Table 3 Variance component estimates for each of the seven diurnal samples.

Sample	A	C	E
At visit			
ACE	.29 (.00-.43)	.00 (.00-.22)	.71 (.57-.89)
AE	**.29 (.14-.43)**	–	**.71 (.57-.86)**
CE	–	**.16 (.07-.26)**	**.84 (.84-.93)**
E	–	–	1.00
11:00 h			
ACE	.00 (.00-.29)	.08 (.00-.18)	.92 (.71-1.00)
AE	.13 (.00-.29)	–	.87 (.71-1.00)
CE	–	.08 (.00-.18)	.92 (.82-1.00)
E	–	–	**1.00**
15:00 h			
ACE	.00 (.00-.26)	.09 (.00-.19)	.91 (.74-1.00)
AE	.13 (.00-.28)	–	.87 (.72-1.00)
CE	–	.09 (.00-.19)	.91 (.81-1.00)
E	–	–	**1.00**
20:00 h			
ACE	.04 (.00-.36)	.12 (.00-.23)	.84 (.64-.95)
AE	**.22 (.07-.37)**	–	**.78 (.63-.93)**
CE	–	**.14 (.05-.23)**	**.86 (.77-.95)**
E	–	–	1.00
22:30 h			
ACE	.00 (.00-.28)	.11 (.00-.21)	.89 (.72-.98)
AE	**.16 (.00-.32)**	–	**.84 (.68-.99)**
CE	–	**.11 (.02-.21)**	**.89 (.79-.98)**
E	–	–	1.00

Sample	A	D	E
Awakening			
ADE	.33 (.10-.53)	.00 (.00-.16)	.67 (.47-.89)
AE	**.33 (.12-.53)**	–	**.67 (.47-.89)**
E	–	–	1.00
Awake+00:30 h			
ADE	.05 (.00-.59)	.44 (.00-.72)	.51 (.28-.94)
AE	**.34 (.03-.61)**	–	**.66 (.39-.97)**
E	–	–	1.00

to a model including only additive genetic and non-shared environmental influences (AE model). Next, we tested whether both awakening samples are influenced by a single common genetic component, or whether additional genes come into play 30 min post-awakening. Removing the genetic effects unique for cortisol levels at 30 min post-awakening (path a_2 in Fig. 3) did not significantly worsen the statistical fit of the model, which indicates that one common genetic component influenced cortisol concentration at both sampling times. The genetic correlation (rG) therefore is 1.00 in this most parsimonious model. Path coefficients from this common genetic factor to cortisol

at awakening and cortisol 30 min post-awakening were similar at .52.

We further tested whether non-shared environmental effects on awakening cortisol levels also influenced cortisol levels 30 min later. Setting the appropriate path (path e_{12} in Fig. 3) to zero significantly reduced the fit of the model, indicating that a significant amount of non-shared environment influences both cortisol levels at awakening and at 30 min post-awakening. In the most parsimonious model, additive genetic components accounted for 34% of the variance of cortisol at awakening and for 32% of the variance of cortisol levels 30 min post-awakening. In this present model, the heritability of the CAR was also
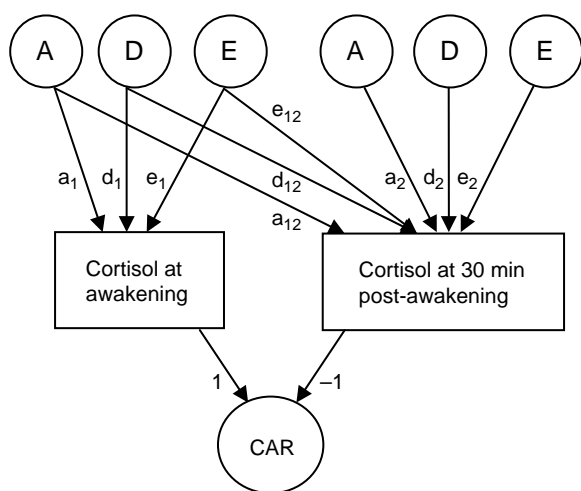
Figure 3 Path diagram of the bivariate model for cortisol during the early morning period. A, additive genetic component; D, dominance genetic component; E, non-shared environmental component; CAR, cortisol awakening response. Letters along the paths represent path coefficients. Subtracting the cortisol concentration at awakening from the concentration 30 min later is established by setting the path coefficients originating from the two measured concentrations (and pointing towards CAR) to 1 (awakening) and −1 (30 min post-awakening). The heritability for CAR in the AE model is computed following the formula.

$$\frac{(a_1)^2 + (a_2)^2 - (2a_1a_{12})}{((a_1)^2 + (a_2)^2 - (2a_1a_{12})) + ((e_1)^2 + (e_2)^2 - (2e_1e_{12}))}$$

estimated. The common genetic factor may influence the second variable in a different degree than the first variable, even though all influence-exerting genes are shared. In that case, this is reflected in a difference in path coefficients, and a significant heritability for CAR. However, model fitting showed that there were no additional genetic factors influencing the CAR when heritability for the two mean cortisol levels is taken into account.

Table 4 shows the variance decomposition results of the bivariate analysis. Table 5 presents the accompanying estimates of genetic and environmental influences under the best fitting model.

4. Discussion

To understand the underlying sources of individual variation in basal cortisol levels throughout the day, we analyzed cortisol data collected at seven fixed time points during the day in adult twins and their singleton siblings from 310 twin families. Results showed that the early morning cortisol concentrations were under considerable genetic control (34–32%), while later daytime samples (11:00–22:30 h) were predominantly under environmental control.

Our finding that the early morning cortisol concentration is under genetic control concurs in part with previous findings. Wüst et al. (2000a) reported significant heritabilities (40 and 48%) for the mean increase and area under the curve of the cortisol awakening response, but not for the awakening sample. Bartels et al. (2003a) showed that genetic factors influenced the awakening sample for 22–24%, and the morning sample taken an hour after awakening for 56–59%. Our current results confirm the presence of significant genetic contributions to the variance of both cortisol levels at awakening and at 30 min post-awakening.

The twin correlations for the early morning samples (awakening and 30 min post-awakening) indicated that dominance genetic effects might influence cortisol levels in the early morning period but model fitting showed that the more parsimonious AE model was preferred over the ADE model. It should be noted that the statistical power to reliably detect genetic dominance effects is small

Table 4 Bivariate model fitting results for cortisol in the early morning period.

Model	−2LL	df	$\Delta\chi^2$	Δdf	Versus	p-value	AIC
ADE	2112.305	868					
AE	2113.148	871	0.843	3	ADE	0.839	−5.157
Reduced AE[a]	**2113.861**	**872**	**0.713**	**1**	**AE**	**0.398**	**−1.287**
Reduced AE[b]	2135.244	872	22.096	1	AE	0.000	20.096
E	2127.868	874	14.72	2	AE	0.002	10.72

−2LL, twice the negative log likelihood; df, degrees of freedom; AIC, Akaike's Information criterion. When the increase in χ^2 ($=\Delta\chi^2$) is not significant (p > .05), the most restrictive model is accepted. **Bold** indicates the most parsimonious model.
[a] No non-shared additive genetic component for cortisol 30 min post-awakening.
[b] No non-shared environmental correlation between cortisol at awakening and cortisol 30 min post-awakening.

Table 5 Variance component estimates for the cortisol measures of the early morning period.

Sample	A	D	E
Awakening	.34 (.13-.53)	–	.66 (.47-.87)
Awakening + 00:30 h	.32 (.05-.59)	–	.68 (.41-.95)
CAR	.00 (0.00-.18)	–	1.00 (.82-1.00)

The estimates for the best fitting model (reduced AEa; i.e. without non-shared additive genetic component) are presented in this table. The 95% confidence intervals are given in parentheses.

(Posthuma and Boomsma, 2000). For the first morning sample (taken during the researcher's visit), the MZ correlation was exactly twice the DZ correlation and no genetic dominance effects were suggested. Model fitting showed that a model including familial factors, most likely genetic, was the preferred model. In contrast to Wüst et al. (2000a), we did not find a genetic influence for the cortisol awakening response (CAR). Our bivariate analysis showed that the genetic influence on cortisol levels at awakening and at 30 min post-awakening completely overlapped. As a result, no additional heritability for the CAR was found.

Our results indicate that the variation in daytime cortisol levels, in particular during the late morning and afternoon, is predominantly influenced by non-shared environmental factors. The large impact of non-shared environmental factors on cortisol levels from late morning to evening agrees with the notion that cortisol is secreted as a reaction to disturbance in the homeostatic equilibrium. Whether shared environmental factors play an additional role remains unclear. Like Bartels et al. (2003a), the study lacked power to discriminate between the AE and CE model for some of the sampling times, although the power in our study was larger than in all previous studies. The extended twin design employed in the current study increases statistical power to distinguish between the components A, C and E compared to a design including only MZ and DZ twins, giving it a statistical power sufficient to reliably detect familial effects larger than 35%. For effects smaller than 35% over 1500 subjects are needed (Posthuma and Boomsma, 2000).

Recently, several studies reported on the effect of birth weight on cortisol concentrations (Phillips et al., 2000; Kajantie et al., 2002, 2004). The lower birth weight in twins may impact, according to the 'Barker hypothesis', on HPA axis activity (Phillips et al., 2000). In the present study, we were able to test whether the results obtained in twins differed from those in singleton siblings. By comparing

singleton siblings with twins from the same family, the two comparison groups are perfectly matched for familial influences (same parents, different intrauterine circumstances, same family environment). Our analyses showed that MZ and DZ twins and singleton siblings did not differ from each other in means or variances on any of the basal diurnal cortisol samples. Importantly, sibling-sibling covariance did not differ from sibling-twin or DZ-twin covariance, which strongly argues against a special twin intrauterine disadvantage with deleterious effects on adult cortisol concentrations. The lower birth weight in twins therefore does not seem to be a sign of diminished growth in the womb, but seems a natural adaptation to a twin pregnancy. The absence of any twin-singleton difference further indicates that estimates of the heritability of cortisol from twin studies generalize to the population at large.

The availability of electrocardiogram and movement registration allowed us to check whether the time the awakening sample was taken corresponded with the real awakening time. Our results indicated that the observed negative awakening response in a subset of subjects was most likely due to an earlier awakening time than reported, resulting in an earlier acrophase than assumed based on the reported sampling times. These results suggests we should be careful when dealing with deviant cortisol awakening responses, as a decreased or altered response might as well be an artifact due to an earlier awakening (Desir et al., 1981; Smyth et al., 1997; Kunz-Ebrecht et al., 2004). The same care should be taken when examining cortisol levels at other times throughout the day. These observations should encourage future studies to very carefully check whether the first morning samples are taken at the moment subjects actually awake, not when they are about to get up. A way of checking the compliance of subjects is by an electronic monitoring device attached to the salivette that accurately time stamps the moment the salivette was used (Kudielka et al., 2003). Although this device is very helpful in the precise determination of the sampling times, it cannot detect that a subject awakes, but does not take the awakening sample until later on. Additional ECG and/or motility recording of the night and early morning hours can provide the true awakening time.

An explanation for the varying genetic influences found for basal cortisol may lie in the difference in the role of the HPA axis and cortisol in the early morning hours and during the day. In the early morning hours, the biological clock of our body, the suprachiasmatic nucleus, prepares the body for the

upcoming period of activity by an anticipatory rise in among others heart rate and cortisol (Buijs et al., 2003). As shown by our and other results, genetic factors control the absolute values of cortisol levels in this awakening period. During daytime, the main objective of the HPA axis is to maintain homeostasis within the body. Therefore, the absolute daytime levels of cortisol will be controlled by environmental feedback. This overshadows any genetic influence on the individual differences in cortisol during the day.

Up until now gene finding studies for basal cortisol have focused on either baseline cortisol measured anytime between 8:00 and 17:00 h (e.g. Baghai et al., 2002; Keavney et al., 2005)), or on total diurnal cortisol (e.g. Rosmond et al., 2001). The current study shows that, while cortisol levels in the awakening period are influenced by genetic factors, cortisol levels throughout most of the day are not heritable; indicating that future gene finding studies for basal cortisol should exclusively focus on the awakening period.

Acknowledgements

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