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Heritability of Daytime Cortisol Levels in Children

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Individual differences in the level of the stress hormone cortisol play a prominent role as an explanatory variable in studies on psychopathology. Relatively few studies have paid attention to individual differences in cortisol levels and the etiology of these differences, in particular their possible genetic basis. All these studies have been in adults. The aim of this study was to estimate genetic and environmental influences on basal cortisol levels in 12-year-old children. To this end, four samples of salivary cortisol were collected on two consecutive days in a sample of 180 twin pairs. Low correlations were found between cortisol levels at different points in time during the day. A significant genetic contribution was found to the variation of basal cortisol levels in the morning and afternoon samples, but not in the evening sample. Heritability did not differ for boys and girls and was highest (60%) for cortisol levels during the sample taken about 45 minutes after awakening. This cortisol awakening response provides a useful endophenotype in the search for genes that may affect hypothalamic-pituitary adrenocortical functioning in children.

KEY WORDS: Basal cortisol; twin study; genetics; circadian rhythm.

INTRODUCTION

Cortisol is a steroid hormone secreted by the outer cortex of the adrenal gland. Its secretion is stimulated by ACTH (adrenocorticotrophic hormone), produced in the pituitary in response to corticotropin-releasing hormone (CRH), a product from neurons in the paraventricular nucleus of the hypothalamus. After its release, the major part of cortisol binds to the plasma proteins corticosteroid binding globulin (CBG, or transcortin) and albumin, which prevents the hormone from penetrating the membranes of their target cells. About 3–5% of the total cortisol is the unbound, biologically active fraction. This active fraction has permissive, suppressive, stimulatory, and preparative action effects in the realms of cardiovascular function, fluid volume and hemorrhage, immunity and inflammation, metabolism, neurobiology, and reproductive physiology (Sapolsky, Romero, and Munck, 2000). Although cortisol is mainly known for its pivotal role in generating an adequate response to physical and emotional stressors, it may also exert strong behavioral effects that are already apparent during childhood. Many studies have reported an association between cortisol levels and Internalizing and Externalizing problem behaviors in children (McBurnett *et al.,* 2000, 1996, 1991; Dawes *et al.,* 1999; Van Goozen *et al.,* 1998; Scerbo and Kolko, 1994; Vanyukov *et al.,* 1993; Tennes and Krey, 1985; Dorn *et al.,* 1999; Granger *et al.,* 1994; Scerbo and Kolko, 1994; McBurnett *et al.,* 1991; Kagan *et al.,* 1987; Tennes *et al.,* 1986). An obvious question for behavior geneticists, therefore, is whether the known genetic contribution to these problem behaviors is partly mediated through genetic effects on the hypothalamic-pituitary adrenocortical (HPAC) axis generating this important stress hormone.

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In the characteristic diurnal rhythm of plasma cortisol level, typically 10–15 well-defined pulses of variable amplitude are observed, with a morning maximum, declining levels throughout the daytime, a period of low concentrations generally centered around midnight, and an abrupt rise after the first few hours of sleep (Weitzman, 1971). Within the first 30 minutes after awakening, free cortisol levels rise by 50–60% (Pruessner *et al.,* 1997; Wüst *et al.,* 2000). Plasma cortisol release is tightly regulated through negative feedback at the pituitary, hypothalamus, and hippocampus (Kovacs *et al.,* 1987; Jacobson and Sapolsky, 1991). This negative feedback is mediated via two types of adrenal steroid receptors: the high-affinity mineralocorticoid receptors (MR) in the hippocampus and the low-affinity glucocorticoid receptors (GR) widely distributed throughout the brain. Strength of this feedback signal strongly varies with time of day (Dorin *et al.,* 1996; Huizinga *et al.,* 1998; Young *et al.,* 1998), contributing to the characteristic diurnal rhythm in plasma cortisol levels (see Fig. 1). Because the activated GR and MR receptors act as transacting factors (Meyer, de Kloet, and McEwen, 2000), it is likely that genetic variation in the *cis*-acting elements for these activated receptors can act to create significant individual variation in diurnal cortisol profiles. However, genetic variation in cortisol levels may also arise at many other points in the HPAC axis, for instance, in the synthesis of corticotrophin-releasing hormone (CRH) or ACTH or in the production of their receptors or those that code for mineralocorticoid (MR, or Type-I receptor) and the glucocorticoid (GR, or Type-II receptor) receptors themselves. In animal studies, polymorphism(s) in the latter gene have already been associated with various aspects of cortisol metabolism such as varying basal cortisol levels (Rosmond *et al.,* 2000a; Rosmond *et al.,* 2000b) and differences in sensitivity to glucocorticoids (Huizinga *et al.,* 1998).

Twin studies constitute a powerful method for identifying genetic influences on (diurnal changes in) cortisol levels in humans. Surprisingly few attempts have been made to estimate the relative impact of genetic and environmental factors on the regulation of cortisol levels (for a review, see Bartels *et al.,* 2003). Most of the 11 reviewed studies (Young *et al.,* 2000; Wüst *et al.,* 2000; Froelich *et al.,* 2000; Pritchard *et al.,* 1999; Inglis *et al.,* 1999; Pritchard *et al.,* 1998; Linkowski *et al.,* 1993; Kirschbaum *et al.,* 1992; Meikle *et al.,* 1988; Nurnberger *et al.,* 1982; Maxwell *et al.,* 1969) point to the direction of moderate genetic contributions to different aspects of cortisol measures.

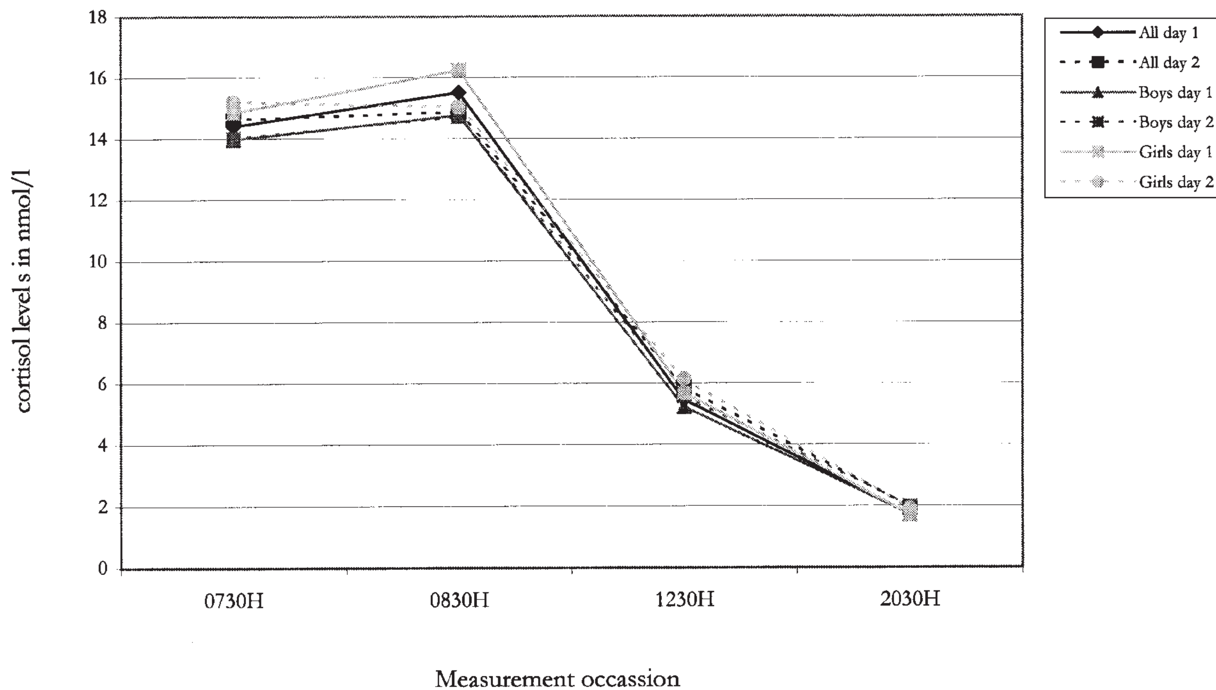

Fig. 1. Graphical representation of the mean cortisol levels (nmol/L) at each measurement occasion for the total sample and boys and girls separately.

Thus, Maxwell and colleagues (1969) showed a significant smaller intrapair variance in MZ as compared to DZ female twin pairs. Meikle *et al.,* (1988) reported evidence of moderate genetic effects ($h^2 = 51\%$) on basal cortisol levels in males. More recently, Inglis *et al.,* (1999) has reported a heritability of 46% in morning plasma cortisol samples. Furthermore, significant heritabilities have been found in the cortisol stress response and in the cortisol response to awakening (Kirschbaum *et al.,* 1992; Wüst *et al.,* 2000). The main problem plaguing many of these studies is the relatively low number of twin pairs (varying from 12 to 146 twin pairs) on whom cortisol was obtained. A power analysis revealed that none of the 11 studies examined in the review paper consists of a large-enough sample size to be able to separate genetic and environmental influences. A combined analysis of five comparable studies (Wüst *et al.,* 2000; Froehlich *et al.,* 2000; Inglis *et al.,* 1999; Linkowski *et al.,* 1993; Meikle *et al.,* 1988) estimated a heritability of 62% for basal cortisol levels with a combined sample size of 399 twin pairs (209 MZ and 190 DZ).

The obvious approach to increase power is to increase the sample size. The recent development of large twin registries all over the world (e.g., Boomsma, 1998) and of ambulatory salivary sample collection methods (Aardal and Holm, 1995; Kirschbaum and Hellhammer, 1994; Riad-Fahmy *et al.,* 1982) has made it more feasible to measure on large numbers of subjects relatively easily in the future. Actual resources of time, money, and practical attainability now mostly restrict the sample size. The power of twins studies to detect additive genetic or environmental variation, however, can be increased through other means besides increasing the sample size (Cohen, 1977; Neale *et al.,* 1994). Relevant for the present study is that an increase in power can be achieved through multivariate analyses—for instance, by a repeated measurement design. Provided that those repeated measurements correlate significantly with each other, this yields large gains in power (Schmitz *et al.,* 1998). In this multivariate method not only is the expectation for the within-pair covariances taken into account but also the cross-trait as well as the within-person information. A method to obtain repeated measures on basal cortisol is to repeatedly sample across an entire measurement day.

A practical strategy would be to sample repeatedly on a single measurement day. However, there are large changes in mean and variance due to the circadian rhythm. It is entirely possible that the contribution of genetic variance changes across the day, possibly due to the expression of different genes. From a physiological "content" point of view, it is valuable per se to assess the genetic architecture of cortisol level at different points of the diurnal curve, i.e., to understand the sources underlying individual variation in the morning peak level as well as through the evening. The optimal approach, therefore, is to sample the same time point across multiple days instead of multiple time points on a single measurement day.

Full understanding of the genetic architecture of basal cortisol level awaits studies with large twin samples that measure cortisol repeatedly at fixed time points from the awakening time and do so on repeated days. Moreover, all our current knowledge of the genetics of cortisol comes from studies in adults. Estimates of the strength of genetic and environmental influences on variation in basal cortisol levels obtained in adults cannot be generalized to children. The developmental trajectories of the various steroid hormones are intertwined and points of cross-talk between the HPAC axis and the gonadal hormones have been shown (Vamvakopoulos and Chrousos, 1994). Just by considering the large changes in gonadal hormone levels from childhood to adolescence, it would be unwise to extrapolate adult genetic architecture of cortisol levels (which itself is unlikely to be stable across the adult life span) to preadolescent children. Indeed, for the behavioral phenotypes possibly influenced by cortisol, cognitive ability, and problem behavior, a change in the strength of genetic and environmental influences throughout development has already been observed (Bartels *et al.,* 2002a, 2002b). So, insight into the cause of individual differences in basal cortisol levels in childhood, besides the current knowledge in adults, is essential.

The aim of this study is to determine the heritability of variation in daytime cortisol levels in children. In accordance with the methodological consideration mentioned above, we collected saliva samples at four fixed points of time on two consecutive days in a large group of 12-year-old twins.

METHODS

Subjects

This project is part of an ongoing, longitudinal study on the development of cognition and emotional and behavioral problems in children. The sample was obtained from the Netherlands Twin Register. The Netherlands Twin Register (NTR), kept by the Department of Biological Psychology at the Vrije Universiteit

in Amsterdam, was established in 1987 (Boomsma *et al.,* 1992; Boomsma, 1998, Boomsma *et al.,* 2002). Young twins and multiples are recruited a few weeks or months after their birth. Currently around 50% of all newborn multiples in The Netherlands are registered. The initial sample of 209 twin pairs was selected on the basis of age and zygosity of the twins and their city of residence. Details on the demographic characteristics of the sample and information on parental occupation can be found elsewhere (Rietveld *et al.,* 2000). For the determination of cortisol levels, saliva was collected in 1999/2000 when the twins were 12 years old. Mean age of the subjects was 12 years (80% ranging from 11 years and 11 months to 12 years and 1 month). Zygosity of the same-sex twins was established by either blood group polymorphisms or DNA analyses. The initial twin sample at age 12 consisted of 47 monozygotic female (MZF), 37 dizygotic female (DZF), 42 monozygotic male (MZM), 44 dizygotic male (DZM), and 39 dizygotic pairs of opposite sex (DOS). Because of difficulties during saliva collection or laboratory analyses, data of 29 twin pairs were not usable, resulting in a final sample of 180 twin pairs. The exact numbers of cortisol samples for each point in time can be found in Table I. Pubertal status has been determined by self-report of the Tanner scales (Marshall and Tanner, 1969, 1970). This scale consists of drawings of breast and pubic hair for girls and drawings of genitalia and pubic hair for boys. The drawings are classified into five stages of development from preadolescent to mature stage. Additionally, girls were asked if they menstruate.

Saliva Collection

Four samples of cortisol per day on two consecutive days were collected using the Salivette sampling device (Starstedt, Rommelsdorf, Germany). Salivary cortisol measurements reflect the biologically active free form. Salivary free cortisol is approximately 70% of that of serum free cortisol because of

Table I. Descriptive Statistics for the Total Sample and for Boys and Girls Separately

							Skewness		Kurtosis	
		N^a	Min	Max	Mean	Std		*s.e.*		*s.e.*
Day 1—0730H	all	309	5.42	27.86	14.40	4.66	.566	.139	−.211	.276
	♂	158	5.78	27.35	13.97	4.66	.675	.193	.165	.384
	♀	151	5.42	27.86	14.85	4.62	.476	.197	−.216	.392
Day 1—0830H	all	324	2.70	36.17	15.52	6.69	.672	.135	.174	.270
	♂	158	2.70	33.15	14.76	6.15	.615	.193	.165	.384
	♀	166	4.01	36.17	16.24	7.10	.646	.188	.015	.375
Day 1—1230H	all	315	2.38	10.19	5.46	1.66	.429	.137	−.465	.274
	♂	155	2.43	10.19	5.23	1.67	.674	.195	.049	.387
	♀	160	2.38	9.50	5.69	1.63	.224	.192	−.749	.381
Day 1—2030H	all	293	.59	3.98	1.73	.67	.892	.142	.395	.284
	♂	150	.59	3.78	1.73	.67	.892	.198	.425	.394
	♀	143	.62	3.98	1.72	.66	.901	.203	.418	.403
Day 2—0730H	all	317	6.07	24.96	14.62	4.53	.323	.137	−.759	.273
	♂	158	6.07	24.96	14.02	4.31	.549	.193	−.317	.384
	♀	159	6.13	24.96	15.20	4.68	.106	.192	−.969	.383
Day 2—0830H	all	293	5.14	27.99	14.87	5.44	.388	.142	−.671	.284
	♂	146	5.14	26.51	14.71	5.25	.417	.201	−.628	.399
	♀	147	5.23	27.99	15.03	5.65	.356	.200	−.714	.397
Day 2—1230H	all	309	2.07	11.27	5.87	1.93	.445	.139	−.337	.276
	♂	149	2.30	10.64	5.58	1.81	.499	.199	−.325	.395
	♀	160	2.07	11.27	6.15	2.01	.355	.192	−.386	.381
Day 2—2030H	all	296	.53	4.87	1.95	.89	.961	.142	.619	.282
	♂	142	.55	4.79	1.99	.86	.912	.203	.762	.404
	♀	154	.53	4.87	1.91	.92	1.028	.195	.603	.389

[a] Number of subjects.

conversion of cortisol to cortisone in the salivary glands. However, salivary cortisol levels correlate very strongly with plasma free cortisol (Aardal and Holm, 1995; Kirschbaum and Hellhammer, 1994; Riad-Fahmy *et al.,* 1982).

Salivettes were sent to the participants by mail and the twin pairs collected their saliva at home, following a written instruction. The samples were collected at prescribed times and, importantly, at the same time for both children of a twin pair. On the first day the first sample (day 1—0730H) was taken in the morning just before getting up (still lying in bed) (mean time 0728H), the second (day 1—0830H) sample was taken at least half an hour after getting up but before going to school (mean time 0817H), the third sample (day 1—1230H) was taken before lunch (mean time 1234H), and the fourth sample (day 1—2030H) was taken in the evening (mean time 2032H). On the second day the same schedule was adapted for four repeated samples. The twins were instructed to collect saliva on two school days to restrict the awakening time and time of sampling. School starting time and lunch break is at approximately the same time all over the Netherlands, resulting in small sampling-time variation. Each participant was asked to write down the exact sampling time in a time schedule and to note exceptional events interfering with daily routine. Subjects were instructed not to brush their teeth before completing saliva sampling to avoid contamination of saliva with blood caused by micro-injuries in the oral cavity. Also, subjects were instructed to thoroughly rinse their mouth with tap water before sampling saliva and not to eat sour food or drink aerated drinks. Subjects were strictly instructed to collect saliva before taking lunch at time point 3. Saliva samples were stored in the freezer until completing the experimental protocol and the samples were picked up by the test administrator and sent by courier to the laboratory in Germany (Trier and Düsseldorf).

Saliva Sampling

The saliva samples of twins of the same pair were randomly distributed over different batches, but the samples of a single subject were placed in one batch. The analyses were performed without knowledge of the zygosity of the twins and without knowledge of exact time of collection. Saliva samples were spun at 3300 rpm for 5 minutes, and cortisol in saliva was determined by time-resolved fluorescence immunoassay, as described elsewhere (Dressendörfer *et al.,* 1992; Wüst *et al.,* 2000). Intra- and interassay variability of the assay was less than 10 and 12%, respectively.

Data Analyses

Descriptive statistics for each sample were calculated using SPSS/Windows 10.0. Pearson correlations were used to test the association between the samples collected on the same day and the association between the samples taken at the same point in time on the two consecutive days. MZ and DZ cross-correlations and twin correlations for the five zygosity groups (MZM, DZM, MZF, DZF, DOS) have been calculated to get a first impression of the genetic and environmental influences on salivary cortisol levels at the different points in time. The cross-correlations represent cross-day–cross-twin correlation and in that matter represents the repeated measurement design. For instance, sample 1 at day 1 for the oldest of the twin is correlated with sample 1 at day 2 for the younger twin.

Genetic Modeling

Genetic model fitting of twin data allows for separation of the observed phenotypic variance into its genetic and environmental components. Additive genetic variance (A), is the variance that results from the additive effects of alleles at each contributing genetic locus. Shared environmental variance (C) is the variance that results from environmental events common to both members of a twin pair. Unique environmental variance (E) is the variance that results from environmental effects that are not shared by members of a twin pair. Estimates of the unique environmental effects also include measurement error. To account for this source of variance, E is always specified in the model.

The different degree of genetic relatedness between monozygotic (MZ) and dizygotic (DZ) twin pairs was used to estimate the contribution of these factors to the phenotypic variation in cortisol levels (Plomin *et al.,* 1997). Similarities for MZ twins are assumed to be due to additive genetic influences plus environmental influences that are shared by both members of a twin pair. Experiences that make MZ twins different from one another are unique environmental influences. Because DZ twins share 50% of their genetic material on average, like other siblings, genetic factors contribute only half to their resemblance. As for MZ twins, the shared environment contributes fully. Model fitting to twin data is based on the comparison of the variance–covariance matrices in MZ and DZ twins. Exploiting the known difference in genetic contribution to intra-pair resemblance of MZ and DZ twin pairs, influences of additive genetic, shared environmental, and unique

environmental factors are estimated using the computer program Mx (Neale *et al.,* 1999).

Per time point, a bivariate model (Cholesky decomposition), based on cortisol samples from the same point in time on the two consecutive days, was used to estimate genetic and environmental influences (see Fig. 2). Rather than decomposing the variance of a single cortisol sample into genetic and environmental sources of variance, bivariate genetic analysis decomposes the variance of each sample and the covariance between the samples at the same time on the two measurement days into genetic and environmental sources.

To make optimal use of all available data, including incomplete twin pairs, analyses were performed on the raw data. In Mx the handling of such "incomplete" data is implemented by calculating twice the negative log-likelihood (−LL) of the raw data of each twin pair and summing these over all pairs. When two models, which provide −2LLs, are nested, subtracting the two −2LLs from each other provides a (−2LL), which has a χ^2 distribution. A high χ^2 against a low gain of degrees of freedom (*df*) denotes a worse fit of the second, more restrictive model relative to the first model. If no significant difference is observed, the more parsimonious model is preferred.

We began with fitting an ACE model. The issue of possible sex differences in heritability or environmental influences is sorted out in the model-fitting procedures. First, we tested whether different genes influence basal cortisol levels in boys and girls or whether the same or different shared environmental factors influence cortisol levels in boys and girls. It was also tested whether the influences of the genes are of different strength in boys and girls. Significance of genetic and shared environmental influences was tested. To this end, it was tested whether a model with additive genetic and unique environmental influences only (AE) gave a significantly worse fit than the full model (ACE). It was also tested whether a model with shared environmental influences and unique environmental influences (CE) gave a significantly worse fit than the full model (ACE). Finally, it was tested whether individual differences of cortisol levels are based on unique environmental influences solely (E model). Estimates of genetic, shared environmental, and unique environmental influences on basal cortisol levels at each point of time separately have been estimated based on the best-fitting model.

RESULTS

Descriptive statistics of the cortisol measures assessed during the day are presented in Table II. Means and standard deviations have been calculated for the

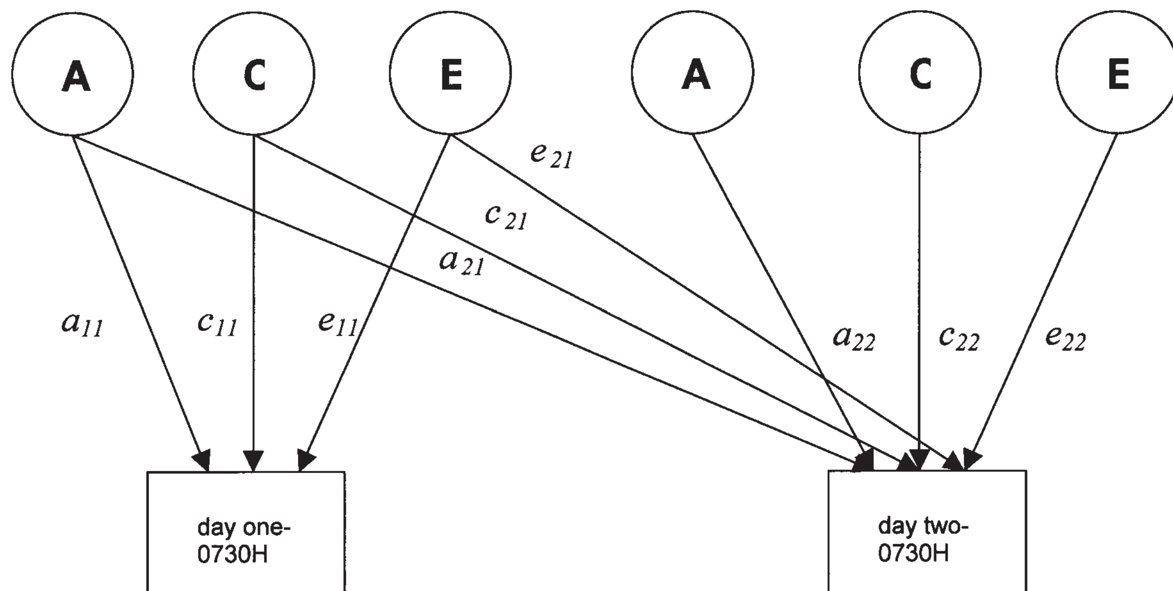

Fig. 2. Cholesky decomposition model for a sample on the first day and a sample on the second day at the same time.

entire sample and for boys and girls separately. Skewness and kurtosis showed that the variables were approximately normally distributed, so no transformation was conducted. Figure 1 shows the expected circadian rhythm with an increase of cortisol levels in the morning and decreasing levels over the day. No significant differences for boys and girls are observed.

Information on pubertal status was collected for 172 girls and 160 boys using the Tanner self-report. For girls the distribution over the stages 1 to 5 (for details, see Marshall and Tanner, 1969) for breast size is 21.8% stage 1, 37.9% stage 2, 28.7% stage 3, and 11.5% stage 4. No girl in our sample reached stage 5 (mature) for breast size. For pubic hair the distribution in girls is 27.3% stage 1, 23.8% stage 2, 27.3% stage 3, 19.2% stage 4, and 1.1% stage 5; 13.5% of the girls did have their first menarche before the day of saliva collection. For boys the distribution over the stages 1 to 5 (for details, see Marshall and Tanner, 1970) for genitalia growth is 15% stage 1, 16.9% stage 2, 50% stage 3, 16.9% stage 4, and 1.3% stage 5. For pubic hair the distribution in boys is 52.1% stage 1, 43.6% stage 2, 2.5% stage 3, 1.8% stage 4. No boy in our sample reached stage 5 (mature) for pubic hair. As expected, girls are more mature than boys and only a small part of the sample is fully mature at the age of 12. No significant influence of pubertal status on cortisol levels could be observed.

Phenotypic correlations for cortisol levels are presented in Table II. Very low associations are found between samples taken on the same day at different time points. Significant correlations are found between samples taken at the same point in time on the two consecutive days (boldfaced). The MZ and DZ cross-correlations, presented in Table III, suggest influences of genetic factors on the association between the two same samples on the two different days (boldfaced). Twin correlations for the five zygosity groups separately are presented in Table IV. As for the MZ and DZ correlation, these twin correlations suggest genetic influences on sample 1 (0730H), sample 2 (0830H), and sample 3 (1230H). Individual variation for sample 4 (2030H) is mainly due to environmental factors.

The results of the bivariate model-fitting procedure for each time point demonstrate different contributions of genetic and environmental influences at the four cortisol measures (Table V). However, no significant sex differences have been found. For sample 1 (0730H) no clear distinction could be made between genetic or shared environmental influences as the primary cause of familial aggregation, both model 5 (AE) and 6 (CE) are not significantly different from a model with both A and C present (model 4). Reducing the additive genetic influences to one common influence on day 1 and day 2 did not significantly worsen the fit (model 7). The best-fitting model for sample 2 (0830H) is a Cholesky decomposition model with additive genetic influences and unique environmental influences (model 5). For sample 3 (1230H) the same pattern as for sample 1 was found. The best-fitting model is a model with additive genetic and unique environmental influences (model 7). For sample 4 (2030H) no factors of familial aggregation could be detected. The best-fitting model is a model with unique environmental influences only (model 7).

Unstandardized and standardized estimates of genetic and environmental influences based on the best-fitting models are presented in Table VI. Significant genetic influences are found for sample 1 (22%, 24%), sample 2 (56%, 59%), and sample 3 (30%, 21%). The heritabilities on the two consecutive days show slight differences. This is due to differences in unique

Table II. Phenotypic Cross-Correlations of Cortisol Levels with Their 95% Confidence Intervals

	Day 1—0730H	Day 1—0830H	Day 1—1230H	Day 1—2030H	Day 2—0730H	Day 2—0830H	Day 2—1230H
Day 1—0830H	.02 (−.10−.15)	—					
Day 1—1230H	.21 (.10−.33)**	.22 (.10–.33)**	—				
Day 1—2030H	.03 (−.10–.15)	.03 (−.09–.15)	.09 (−.03–.21)	—			
Day 2—0730H	**.36 (.25–.46)****	.10 (−.02–.21)	.14 (.03–.25)	.10 (−.03–.23)	—		
Day 2—0830H	.01 (−.12–.14)	**.36 (.24–.46)****	.23 (.10–.33)	.02 (−.11–.14)	.03 (−.08–.15)	—	
Day 2—1230H	.07 (−.04–.19)	.19 (.07–.30)	**.24 (.13–.35)****	.14 (.02–.26)	.17 (.05–.28)*	.21 (.09–.32)**	—
Day 2—2030H	−.00 (−.12–.12)	.06 (−.07–.19)	.16 (.04–.27)	**.21 (.09–.33)****	.10 (−.02–.22)	.18 (.05–.29)**	.04 (−.08–.16)

* Correlation is significant at .05 level (two-tailed).
** Correlation is significant at .01 level (two-tailed).

Table III. MZ (above diagonal) and DZ (below diagonal) Correlations and Cross-Correlations

	S11a	S21a	S31a	S41a	S12a	S22a	S32a	S42a	S11b	S21b	S31b	S41b	S12b	S22b	S32b	S42b
S11a	—	.01	.24	.01	.28	−.02	.10	.06	**.33**[a]	.00	.09	.07	**.18**[c]	.00	.00	.03
S21a	.02	—	.30	−.11	.00	.47	.23	.02	.00	**.64**[a]	.16	−.12	−.04	**.46**[c]	.17	.10
S31a	.21	.16	—	.03	.10	.40	.29	.35	.09	.16	**.45**[a]	.00	.23	.35	**.24**[c]	.19
S41a	.03	.17	.14	—	.11	−.18	.15	.18	.07	−.11	−.06	**.12**[a]	.14	−.18	−.11	**.00**[c]
S21a	.41	.21	.17	.11	—	−.03	.20	.11	.18	−.04	.23	.14	**.43**[a]	.03	−.05	.13
S22a	.04	.33	.10	.17	.10	—	.30	.27	.00	.46	.35	−.18	.03	**.62**[a]	.25	.10
S32a	.06	.19	.24	.17	.16	.15	—	.12	.00	.17	.24	−.11	−.05	.25	**.31**[a]	.04
S42a	−.06	.15	.03	.27	.09	.16	−.03	—	.03	.10	.19	.00	.13	.10	.04	**.14**[a]
S11b	**.17**[b]	.08	.13	.01	.08	.06	−.07	.07	—	.00	.24	.01	.28	−.02	.10	.06
S21b	−.08	**.32**[b]	.02	.08	−.02	.20	.08	.03	.02	—	.30	−.01	.00	.47	.23	.02
S31b	.13	.02	**.25**[b]	.04	.18	.03	.02	.09	.21	.16	—	.03	.10	.40	.29	.35
S41b	.01	.08	.04	**.14**[b]	.00	.11	.04	.08	.03	.17	.14	—	.11	−.18	.15	.18
S12b	**.08**[d]	−.02	.18	.01	**.19**[b]	.00	.05	.03	.41	.21	.17	.11	—	−.03	.20	.11
S22b	.06	**.20**[d]	.03	.12	.00	**.36**[b]	.05	−.03	.04	.33	.10	.17	.10	—	.30	.27
S32b	−.07	.08	**.02**[d]	.04	.05	.06	**.15**[b]	−.01	.06	.19	.24	.17	.16	.15	—	.12
S42b	.07	.03	.09	**.08**[d]	.03	−.03	−.11	**.23**[b]	−.06	.15	.03	.27	.09	.16	−.03	—

Note: S11a to S42b refers to the samples of cortisol; S = sample; the first number is the time of sampling and the day (1 to 4); the second number is the day of sample (day 1 and day 2); a is the oldest of the twin and the boy in DOS twins; b is the youngest of the twin and the girl in DOS twins.
[a] Twin correlations for monozygotic twins.
[b] Twin correlations for dizygotic twins.
[c] Cross-correlations for monozygotic twins.
[d] Cross-correlations for dizygotic twins.

environmental influences that change across days because they also contain day-specific measurement error. Since the total variance equals 100%, the differences in unique environmental influences are reflected in the small differences in heritability.

Because the between-time-point (on the same day) correlations were very low, multivariate models with different cortisol samples taken on the same day but at a different point in time were not considered meaningful. If no significant phenotypic correlation is obtained, a significant genetic correlation will be very rare. The only possibility is that there is a positive genetic correlation and a negative environmental correlation, resulting in a nonsignificant phenotypic correlation. If this is the case, considering that only nonshared environmental influences are found and that the subjects are genetically related, the between-subject cross-trait correlations need to be higher than the within-subject cross-trait correlations. In Table 3 no such systematic effect is observed in the data.

Table IV. Twin Correlations for Cortisol Level with Their 95% Confidence Intervals

	MZM[a]	DZM	MZF	DZF	DOS
Day 1—0730H	.28 (.00–.58)	.11 (.00–.42)	.42 (.07–.67)	.00 (.00–.33)	.11 (.00–.46)
Day 1—0830H	.45 (.06–.71)	.34 (.01–.60)	.67 (.44–.82)	.23 (.00–.55)	.14 (.00–.50)
Day 1—1230H	.54 (.19–.75)	.40 (.03–.67)	.43 (.08–.67)	.50 (.13–.73)	.00 (.00–.31)
Day 1—2030H	.00 (.00–.30)	.53 (.10–.76)	.21 (.00–.62)	.13 (.00–.52)	.04 (.00–.44)
Day 2—0730H	.17 (.00–.51)	.00 (.00–.34)	.62 (.36–.79)	.35 (.00–.64)	.18 (.00–.50)
Day 2—0830H	.53 (.17–.76)	.23 (.00–.52)	.68 (40–.83)	.37 (.00–.67)	.35 (.00–.62)
Day 2—1230H	.03 (.00–.43)	.32 (.00–.61)	.31 (.00–.59)	.10 (.00–.49)	.00 (.00–.32)
Day 2—2030H	.00 (.00–.49)	.40 (.04–.66)	.17 (.00–.51)	.19 (.00–.54)	.00 (.00–.27)

[a] MZM = monozygotic males, DZM = dizygotic males, MZF = monozygotic females, DZM = dizygotic females, DOS = dizygotic opposite sex.

Table V. Summary Statistics of the Fit of the Genetic-Environmental Models to the Four Samples of Cortisol Separately

Sample	Model	−2LL	*df*	Model Comparison			
				Comparison	χ^2	Δdf	*p*
0730H	1 Cholesky ACE sex differences r_g DOS free	3616.172	597				
	2 Cholesky ACE sex differences r_c DOS free	3616.153	597				
	3 Cholesky ACE sex differences	3616.153	598				
	4 Cholesky ACE no sex differences	3627.676	607	3	11.523	9	.24
	5 Cholesky AE no sex differences	3628.038	610	4	.362	3	.95
	6 Cholesky CE no sex differences	3630.672	610	4	2.996	3	.39
	7 Cholesky AE $a_{11} = a_{21}$ $a_{22} = 0$	3632.385	612	5	4.347	2	.11
0830H	1 Cholesky ACE sex differences r_g DOS free	3861.988	588				
	2 Cholesky ACE sex differences r_c DOS free	3861.695	588				
	3 Cholesky ACE sex differences	3882.181	589				
	4 Cholesky ACE no sex differences	3870.692	598	3	8.511	9	.48
	5 Cholesky AE no sex differences	3870.922	601	4	.23	3	.97
	6 Cholesky CE no sex differences	3881.929	601	4	11.237	3	.01
	7 Cholesky AE $a_{11} = a_{21}$ $a_{22} = 0$	3882.284	603	5	11.362	2	.00
1230H	1 Cholesky ACE sex differences r_g DOS free	2444.214	595				
	2 Cholesky ACE sex differences r_c DOS free	2442.230	595				
	3 Cholesky ACE sex differences	2445.157	596				
	4 Cholesky ACE no sex differences	2453.593	605	3	8.436	9	.49
	5 Cholesky AE no sex differences	2453.593	608	4	.00	3	1.00
	6 Cholesky CE no sex differences	2457.805	608	4	4.212	3	.24
	7 Cholesky AE $a_{11} = a_{21}$ $a_{22} = 0$	2456.984	610	5	3.391	2	.18
2030H	1 Cholesky ACE sex differences r_g DOS free	1337.975	560				
	2 Cholesky ACE sex differences r_c DOS free	1336.863	560				
	3 Cholesky ACE sex differences	1338.487	561				
	4 Cholesky ACE no sex differences	1341.947	570	3	3.46	9	.94
	5 Cholesky AE no sex differences	1343.412	573	4	1.465	3	.69
	6 Cholesky CE no sex differences	1344.376	573	4	2.429	3	.49
	7 Cholesky E no sex differences	1347.354	576	4	5.407	6	.49

Table VI. Unstandardized Variance Components and Standardized Estimates for Additive Genetic, Shared Environmental, and Unique Environmental Influences with Their 95% Confidence Intervals

Sample	Model	V_T[a]	V_G[b]	V_E[c]	A	C	E
Day 1—0730H	Model 7: AE	21.63	4.74	16.89	.22 (.09–.35)	—	.78 (.65–91)
Day 2—0730H	Model 7: AE	20.08	4.74	15.34	.24 (.09–.37)	—	.76 (.63–91)
Day 1—0830H	Model 5: AE	43.52	24.38	19.13	.56 (.39–.69)	—	.44 (.31–.61)
Day 2—0830H	Model 5: AE	30.05	17.80	12.25	.59 (.42–.72)	—	.41 (.28–.58)
Day 1—1230H	Model 7: AE	2.67	.81	1.87	.30 (.15–.43)	—	.70 (.57–.85)
Day 2—1230H	Model 7: AE	3.77	.81	2.97	.21 (.11–.30)	—	.79 (.70–.89)
Day 1—2030H	Model 7: E	.44	.00	.44	—	—	1.00 (1.0–1.0)
Day 2—2030H	Model 7: E	.78	.00	.78	—	—	1.00 (1.0–1.0)

[a] V_T is the total variance.
[b] V_g is the genetic variance.
[c] V_E is the unique environmental variance.

DISCUSSION

The purpose of this study was to estimate the genetic and environmental influences on the variation in basal cortisol levels at four different time points on two consecutive days in a large sample of 12-year-old children. Although previous studies have all used adult twins, the findings were very much in line with previous findings on the genetic architecture of urinary or salivary cortisol levels (Bartels *et al.,* 2003). A significant genetic contribution to basal cortisol levels was found at three of the four time points sampled. Heritability did not differ for boys and girls and was highest (60%) for cortisol levels during the second sample taken about 45 minutes after awakening. A major contribution of unique environmental factors was found that dominated interindividual variation at all time points, save the second sample.

Wüst and colleagues (2000) found a similar pattern of heritabilities, with a moderate-to-high heritability estimate for the cortisol response to awakening (40%) and low-to-nonsignificant heritability estimates for cortisol samples later that day, where unique environmental influences dominated. As suggested by Wüst *et al.* (2000), sleep is a period of very low differentiation in environmental influences that only kick in fully after awakening and accumulate during the day, giving rise to a gradual increase in environmental variance. This could lead to a shift from genetic to environmental control over individual variation in cortisol levels. We explicitly tested in what way the changes in genetic architecture across time points reflected a change in the ratio of genetic and environmental variance. In contrast to the suggestion by Wüst *et al.,* we found that the relative increase in genetic variance at the second sample compared to the other samples was much more pronounced than the increase in environmental and total variance. Alternatively, therefore, we hypothesize that the heritability of cortisol levels varies inversely with the strength of the negative feedback signal exerted by cortisol at the GR and MR receptors. Changes in the strength of this feedback signal are reflected in changes in the absolute cortisol level, although time-lagged, because the effects of cortisol on the GR and MR receptors are largely genomic.

If our above hypothesis is correct, genetic variation in the GR and MR receptors may be important sources of the genetic variation in morning cortisol levels. Since, these receptors act as *trans*-acting factors (de Kloet, 2000), genetic variation in the *cis*-acting elements for these activated receptors can be a second source of genetic variation. Polymorphism(s) in the GR gene have already been associated with various aspects of cortisol metabolism such as varying basal cortisol levels (Rosmond *et al.,* 2000a; Rosmond *et al.,* 2000b) and differences in sensitivity to glucocorticoids (Huizinga *et al.,* 1998). Mutant forms of the GR gene are also found in patients with primary cortisol resistance (Ruiz *et al.,* 2001). Allelic variation in MR sensitivity is likely to further influence basal cortisol levels, although no evidence has been presented to date.

Many other sources of genetic variation should not be ruled out. These include genes that affect corticotropin-releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) synthesis, the affinity and density of their receptors, and their functionality. For instance, an ACTH receptor gene (Mountjoy *et al.,* 1992) has been localized on chromosome 18 (Gantz *et al.,* 1993) and mutations of this gene might lead to the disturbance of the HPAC-axis function. Further progress in understanding the genetics of individual differences in cortisol levels will be made through pharmacological and knockout studies in animals. However, although large homology probably exists between animals and human HPAC genes, genetic linkage or candidate gene studies in humans may ultimately be required. The high heritability of the cortisol level after awakening suggests that this may be the most useful phenotype to attempt gene finding. One of the huge advantages of a twin sample in gene finding is that observed candidate genes and unobserved genes (estimates of genetic influences through the MZ-DZ comparison) can be simultaneously tested.

High and low basal cortisol levels in children have been associated with Externalizing and Internalizing problem behavior, respectively. From 11 studies on the association between cortisol and Externalizing behavior, 8 studies report a negative association (McBurnett *et al.,* 2000, 1996, 1991; Dawes *et al.,* 1999; Van Goozen *et al.,* 1998; Scerbo and Kolko, 1994; Vanyukov *et al.,* 1993; Tennes and Krey, 1985), 1 study reports a positive association (Gerra *et al.,* 1998), and 2 studies report no difference in cortisol levels between the group with Externalizing behavioral problems and normal controls (Schulz *et al.,* 1997; Kruesli *et al.,* 1989). Children with high levels of cortisol are characterized by inhibition of temperament, higher rates of self-reported depression, parent-reported Internalizing problem behavior, social withdrawal, social anxiety, and social problems. Six of the 9 reported studies on the association of Internalizing-related disorders and cortisol found a positive association (Dorn *et al.,* 1999; Granger *et al.,* 1994; Scerbo and Kolko, 1994; McBurnett *et al.,* 1991;

Kagan *et al.,* 1987; Tennes *et al.,* 1986), while a negative association was found in 2 studies (Moss *et al.,* 1995; Vanyukov *et al.,* 1993). One study reported the finding of no association at all (Tennes and Krey, 1985).

These deviations in basal cortisol during childhood suggest a role for disturbed functioning of the hypothalamic-pituitary adrenocortico (HPAC) axis in the pathogenesis of these behavioral disorders. Some circumstantial evidence for this chain of events exists as significant genetic effects have also been found on problem behavior. For instance, van der Valk observed both genetic effects on problem behavior in a large sample of 3-year-old twins (van der Valk *et al.,* 1998a), as well as stable genetic influences on problem behavior at the ages of 10 and 15 years in biologically related and unrelated adoptees (van der Valk *et al.,* 1998b). Significant influences of genetic factors have been found on stability in problem behavior in a large longitudinal sample of Dutch twins followed from age 3 to age 12 (Bartels *et al.,* 2002b). Comparable results of genetic influences on stability have been found in other studies (Verhulst and van der Ende, 1993; van den Oord, 1994; Koot, 1995; Edelbrock *et al.,* 1995; Schmitz *et al.,* 1995). Although these findings allow a scenario in which genetic influences on HPAC-axis functioning translate to a genetic risk for behavioral problems, the opposite—an effect of genetically determined problem behavior on HPAC-axis functioning—cannot be ruled out. Also, an underlying genetic defect may cause problem behavior as well as deviant HPAC function without a direct causal link between these two (pleiotropy) (Blizard, 1992). Future multivariate modeling of cortisol and problem behavior could resolve this main issue of causality.

Finally, it should be emphasized that cortisol in these children was collected at home. Hence, the results of this study give a good insight into the genetic and environmental influences on *basal* cortisol levels but not on cortisol levels in response to specific physical or emotional stressors. Individual differences in stress reactivity may well be a key factor in the link between HPAC-axis functioning and behavior. Cortisol reactivity to standardized stressors, therefore, would be served by future examination in genetically related children.

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