MARKED INTERINDIVIDUAL variability is characteristic for basal and stimulated activity of the hypothalamus-pituitary-adrenal (HPA) axis. Because chronic dysregulation of HPA activity is related to the onset and course of several stress related disorders (1–3), the identification of relevant sources of this variability has long been an important goal in psychoneuroendocrinological research (4).

In humans, relatively little research has been conducted to estimate the impact of genetic factors on the variability in HPA axis function. Studies of the heritability of basal HPA axis activity consistently suggest a moderate genetic load (5–8). For instance, a recent review and simultaneous analysis of five comparable twin studies reported a heritability of 62% for basal free cortisol (9).

Only a few studies have investigated stimulated HPA axis activity (10–13), and most studies argue against a substantial contribution of genetic factors on variation in stimulated cortisol and ACTH levels. One exception is the study of Kirschbaum et al. (11), which suggests a pronounced influence of genetic factors on variation in salivary cortisol levels after stimulation with 100 μg CRH and a moderate genetic effect on responses to a psychosocial stressor. A considerably larger number of twin studies have investigated the heritability of basal and stimulated heart rates. Depending on the specific study design, varying heritabilities have been reported (for a review see Ref. 14).

The role of context (e.g. see Ref. 15), i.e. the set of variables that define a situation for each individual, has not received sufficient appreciation in psychoneuroendocrine studies assessing the heritability of a trait. Most studies on the heritability of HPA axis function report a separate contribution of genetic and environmental factors and treat the resulting coefficients as relatively fixed numbers. However, it seems very reasonable to assume that genes and environment jointly influence the function of this endocrine system. One way to address this issue is to explicitly manipulate the environmental context and to estimate heritability in different environmental settings (16, 17).

The aims of the present study were to investigate the heritability of HPA axis responses to a moderate psychosocial stressor and determine whether these estimates are context dependent. To manipulate environmental context, participants in this study were repeatedly exposed to the same psychosocial stressor to induce a contextual change from a high anxiety/high novelty situation to a low anxiety/low novelty situation.

Subjects and Methods

Fifty-eight male twin pairs aged 16–24 yr (mean age 18.67 yr, sd 2.29) were recruited for participation in this experiment by mail. Postal addresses of potential twin pairs (pairs of individuals with identical dates of birth, birthplaces, and family names) were supplied by the residents’ registration office (Daten- und Informationszentrum; DIZ) of Rheinland-Pfalz, Germany. As confirmed by subsequent DNA analysis (see below), the sample consisted of 33 monozygotic (MZ) and 25 dizygotic (DZ) twin pairs, each raised together in the same household. Before entering the
study, the absence of acute or chronic health problems was confirmed by a medical exam. All subjects reported being medication free. Subjects were excluded when they self-reported on drug use or on excessive use of alcohol, were shift-workers, or previously participated in a study applying a psychosocial stressor. Smokers were included if they agreed to refrain from smoking on test days. Participants received a modest monetary incentive on completion of the experiment. Written informed consent was obtained from all subjects. The investigation described in this manuscript was conducted in accordance with the guidelines described in The Declaration of Helsinki, and the study protocol was approved by the ethics committees of the University of Trier, Germany, and the Rheinland-Pfalz State Medical Association.

Experimental design

All subjects reported to the laboratory three times at 1-wk intervals. At 45 min before stressor onset (~45 min), a catheter was inserted in an antecubital vein, and subjects were instrumented with monitors for automatic assessment of heart rate. In the following 35-min rest period, subjects remained seated and completed a brief state-anxiety scale (see below). To control for cardiovascular responses due to a shift in posture, subjects were asked to stand up at ~10 min relative to stressor onset. At ~2 min, a saliva sample, an EDTA blood sample, and a serum blood sample were obtained. Subjects were then exposed to the Trier Social Stress Test (TSST), which consists of a free speech and a mental arithmetic task in front of an audience and a camera (18). In a recent meta-analysis, this protocol has been shown to be among those that produce reliably induced endocrine responses in about 70–80% of the subjects at the first exposure in previous studies. Including instruction and a short preparation period, the stressful situation lasted for 15 min. Thereafter, subjects remained in an upright posture for 10 min and again completed the state-anxiety scale. Additional saliva and EDTA blood samples were obtained at 16, 25, 35, 45, 60, 75, and 105 min relative to the stressor onset. To reduce possible environmental variations within twin pairs to a minimum, both members of a pair were always investigated on the same day. Furthermore, to correct for the pronounced circadian variations of HPA axis activity, all TSSTs were applied between 1600 and 1700 h. Except for slight modifications in the free speech and mental arithmetic tasks, the experimental protocol was identical on all 3 test days.

Saliva and blood sampling

Saliva samples were collected with the Salivette sampling device (Sarstedt, Nümbrecht, Germany), stored at room temperature until completion of the session, and then kept at ~20 °C until analysis. After thawing for biochemical analysis, samples were centrifuged for 10 min at 2000 × g and 10 C. EDTA blood samples were immediately stored on ice and were centrifuged within 30 min at 2000 × g and 4 C for 10 min. Plasma was then divided into aliquots and stored at ~20 °C until hormone analysis. The serum blood sample was kept at room temperature for 30 min and was then processed as the plasma samples.

Hormone assays

The fraction of free cortisol in saliva (salivary cortisol) was determined using a time-resolved immunoassay with fluorometric detection, as described in detail elsewhere (20). Total cortisol concentration was measured in plasma samples with an ELISA (Diagnostic Systems Laboratories, Sinsheim, Germany). For both analytes, inter- and intraassay coefficients of variance were less than 12%. ACTH was measured in five plasma samples (~2 to ~35 min and ~105 min) with a chemiluminescence immunoassay (Nichols Institute, Bad Nauheim, Germany) with an intra- and interassay coefficient of variance of less than 4 and 7%, respectively.

Heart rate

Heart rate (HR) was measured at 5-sec intervals using a transmitter belt with wireless connection to a wrist receiver (Polar Sport Tester, Polar Electro, Groß Gerau, Germany). From ~40 min to ~40 min relative to the stressor, HR measurements were collapsed into five data blocks: pre- and post stress seated, pre- and post stress upright posture, and TSST. To avoid errors due to minor delays in the study protocol, data assessed in the first and last 2 min of each block were ignored.

Assessment of manipulation of context

For anxiety ratings before and after the stressful situation, a German version of the state-anxiety scale of Spielberger’s state-trait-anxiety inventory was employed (21, 22). The intensity of a momentary feeling (e.g., “I am tense,” “I feel nervous”) is rated for 20 items on a 4-point scale ranging from 1 (not at all) to 4 (very much so).

Zygosity testing

DNA was isolated from one EDTA whole-blood sample with a commercially available DNA purification kit (Puregene, Gentra, Minneapolis, MN). Zygosity of all pairs was then determined with a PCR protocol (AmpFISTR profiler PCR amplification kit, Applied Biosystems, Foster City, CA). The probability of misclassifying zygosity with this methodology is less than 0.1%.

Statistical analyses

Two-way ANOVAs for repeated measures were computed to reveal day and time, effects for salivary and total cortisol, ACTH, and HR responses to the TSST. To assess the influence of smoking as a possible confounding factor, three-way ANOVAs (day × sample × smoking) were performed. When the Mauchly’s test of sphericity yielded significant results, Greenhouse-Geisser (if < 0.75) or Huynh-Feldt (if > 0.75) corrections were applied (23, 24), and only corrected results are reported. To test for changes in state-anxiety, t tests for paired samples were employed. In case of significant results, effect sizes (ω²) as a measure of explained variance are reported. A χ² test incorporating Yates’ correction for continuity was used to test for the equality of distributions of the dichotomous variables smoking and zygosity, and the ω-coefficient is reported to indicate the strength of the relationship between the two variables. To obtain indices for the response magnitude, area under the response curve (AUCs) with reference to zero were calculated. All correlations are Pearson correlations, if not indicated otherwise.

The difference in degree of genetic relatedness between MZ and DZ twin pairs is commonly used to estimate the contribution of genetic and environmental factors for a trait of interest. Heritability estimation in this study was based on the standard approach of heritability in the broad sense, taking into account the additive genetic component as well as variance components due to genetic dominance and epistasis. This concept assumes no assortative mating and neither gene-environment correlations nor interactions (for details see Ref. 25). Intrapair correlation coefficients (ri) were calculated for MZ and DZ pairs. They take into account the variance between pairs (VB) and within pairs (WW) and were calculated as follows: ri = vb/vb + WW. The contribution of genetic factors (h²) was estimated by doubling the difference in r coefficients of MZ and DZ pairs (26). The contribution of shared environment (c²) was estimated by subtracting the resulting h² from r(MZ) and the contribution of specific environment and measurement error (e²) was obtained by subtracting r(MZ) from 1 (e.g., see Ref. 27, 28). Mathematically, values less than 0 and more than 1 can result for h² and c². These values are not defined and are interpreted as 0 and 1, respectively. A summary of the twin method, the various assumptions, and the plausibility of these assumptions, have been published elsewhere (e.g., see Ref. 29).

Results

On all three test days, TSST exposure resulted in a significant HPA axis response (time effect, salivary cortisol: all F > 42.21, ω² > 0.27; total cortisol: all F > 96.44, ω² > 0.47; ACTH: all F > 92.30, ω² > 0.47; all P < 0.001). As depicted in Fig. 1, cortisol and ACTH responses to this stress protocol decreased significantly across sessions (group effect, salivary cortisol: F1,5.168.5 = 25.75, ω² = 0.19; total cortisol: F1,6.169.0 = 31.46, ω² = 0.23; ACTH: F1,7.168.1 = 13.32, ω² = 0.12; all P < 0.001). A more pronounced response reduction was observed
from the first to the second stress exposure (group effect, salivary cortisol: $F_{1,107} = 22.01, \omega^2 = 0.17$; total cortisol: $F_{1,106} = 22.96, \omega^2 = 0.18$; ACTH: $F_{1,103} = 16.33, \omega^2 = 0.14$; all $P < 0.001$), followed by a distinctly smaller reduction from the second to the third exposure (group effect, salivary cortisol: $F_{1,110} = 5.33, \omega^2 = 0.05, P < 0.05$; total cortisol: $F_{1,108} = 8.91, \omega^2 = 0.08, P < 0.01$; ACTH: n.s.). Despite this response reduction, a moderate correlation between the AUCs of the three sessions was observed for salivary cortisol ($r = 0.52$–0.62), total cortisol ($r = 0.61$–0.78), and ACTH ($r = 0.43$–0.69; all $P < 0.001$), suggesting a stable underlying trait.

A subgroup of 22 subjects were smokers and reported a mean cigarette consumption of 13.09 cigarettes/d (SD 5.81; range 6–30). In line with previous reports (e.g., see Ref. 30), lower TSST responses were observed in smokers (main effect smoking: salivary cortisol: $F_{1,106} = 6.11, \omega^2 = 0.05$; total cortisol: $F_{1,104} = 5.26, \omega^2 = 0.05$; ACTH: $F_{1,98} = 4.30, \omega^2 = 0.04$; all $P < 0.05$). However, a $\chi^2$ test incorporating Yates’ correction for continuity provides evidence for equal distribution of the dichotomous variable smoking on the variable zygosity ($\chi^2 = 0.99$, $\varphi = 0.21$, n.s.). Furthermore, only five MZ and three DZ pairs were discordant with regard to the variable smoking so that smokers were kept in the analysis.

HR increased significantly on all 3 test days (time effect, all $F > 31.30, P < 0.001, \omega^2 > 0.23$), and the increase of HR responses across sessions (group effect, $F_{2,0,168.0} = 5.47, P < 0.01, \omega^2 = 0.06$) was attributable to the differences between the first two sessions only (group effect, $F_{1,0,94.0} = 10.85, P < 0.01, \omega^2 = 0.10$).

To control for possible errors due to a sample consisting of twin pairs and thus of correlated observations, all ANOVAs were reanalyzed after randomly selecting one partner of each pair. With two exceptions, all ANOVAs revealed results that were comparable with the complete sample, suggesting a minor influence of this potential confounder on our data: first, the response reduction from TSST 2 to TSST 3 was no longer significant in salivary ($F_{1,54} = 0.86$, n.s.) and total cortisol ($F_{1,52} = 2.41$, n.s.), and second, the significant effect of smoking on ACTH responsivity disappeared ($F_{1,48} = 1.81$, n.s.). Furthermore, no significant impact of zygosity could be observed when this variable was introduced as a possible confounder to all ANOVAs.

A successful manipulation of environmental context is reflected by pre- and post stress anxiety levels. Increasing state-anxiety levels were observed in response to the first (t$_{512} = 6.62, P < 0.01, \omega^2 = 0.28$) but not to the second and third TSST exposure (both $t < 1.16$, n.s.), suggesting a change from a high anxiety to a low anxiety context.

The focus of the present study was to investigate the heritability of HPA axis responses to psychosocial stress. As depicted in Fig. 2, neither MZ (t$_{112} = 6.62, P < 0.01, \omega^2 = 0.28$) nor DZ pairs (t$_{112} = 0.15$–0.35, n.s.) showed significant intrapair correlations for the TSST 1 AUC in salivary cortisol, total cortisol, and ACTH. However, after repeated stress exposure and with changing context, a considerable increase of MZ intrapair correlations toward significant associations was observed for all three analytes (TSST 2: $r_i = 0.58$, n.s., to $r_i = 70$, $P < 0.01$; TSST 3: $r_i = 0.66, P < 0.05$–0.78, $P < 0.01$), whereas

![Fig. 1. Salivary cortisol, total cortisol, ACTH, and HR responses to three TSST exposures in 1-wk intervals.](image-url)
DZ similarities remained nonsignificant in TSST 2 and TSST 3 ($r_i = 0.04–0.42$, n.s.). A comparable pattern was observed for HR AUC, with moderate intrapair correlations for both MZ ($r_i = 0.56$, n.s.) and DZ ($r_i = 0.41$, n.s.) twins in TSST 1 and increasing correlations for MZ pairs in the second ($r_i = 0.64$, $P < 0.05$) and third ($r_i = 0.72$, $P < 0.01$) exposure. In Table 1, the contribution of genetic factors, shared environment, and specific environment and measurement error is displayed and reveals increasing heritabilities for salivary and total cortisol, ACTH, and HR.

Finally, heritability was estimated separately for each individual time point, and results are reported for responses averaged across sessions. The finding depicted in Fig. 3 suggests that, for every single time point, MZ twins cortisol levels (salivary cortisol: $r_i = 0.41–0.76$; total cortisol: $r_i = 0.54$...
and \( r = 0.77 \) were more similar than the respective DZ twins. Hormone levels (salivary cortisol: \( r = 0.14-0.52 \); total cortisol: \( r = 0.17-0.31 \)). This result also suggests that the impact of genetic factors on variability in salivary and total cortisol is higher when hormone levels are high (samples +16, +25, +35, +45 min) than when hormone levels are low (prestress sample: -2 min and recovery samples: +60, +75, +105 min). Whereas for baseline and recovery phase samples heritabilities did not exceed \( h^2 = 0.56 \) and \( h^2 = 0.84 \) for salivary and total cortisol, respectively, stimulated values varied between \( h^2 = 0.76 \) and \( h^2 = 1 (h^2 = 1.24) \) for salivary cortisol and between \( h^2 = 0.88 \) and \( h^2 = 1 (h^2 = 1.20) \) for total cortisol.

**Discussion**

The present study suggests that, first, HPA axis responses to moderate psychosocial stress are heritable. Although only a modest heritability coefficient was observed for all measures of physiological stress responsivity after the first TSST exposure, heritability estimates increased substantially after the second and third exposure to the same stressor. This change is rather remarkable because the stress protocol was virtually identical on all three study days. Concurrently, the first but not the second and third TSST exposure was associated with a significant increase in state-anxiety, suggesting that, second, the estimates of heritability are context dependent. There is some evidence in our data that supports this notion.

First, our data suggest that this change in heritability is accompanied by a significant decrease in HPA axis responses from the first to the second exposure and to a lesser degree from the second to the third exposure. The observation of decreasing cortisol and ACTH responses to repeated TSST exposure is not new and has been reported consistently for the TSST (31, 32) and other stress protocols (33–35). The implications of the habituation pattern observed in this sample are discussed in a separate report (36). It has been argued that HPA axis response reductions to similar stressors may be ascribed to a reduction in novelty, unpredictability, and uncontrollability of the situation (e.g. see Ref. 4, 37). Thus, whereas the first TSST setting is characterized by a high novelty/high unpredictability/high uncontrollability context, the second and to a higher degree the third TSST are characterized by a low novelty/low unpredictability/low uncontrollability context. Moreover, whereas increasing levels of state anxiety were observed in response to the first TSST exposure, anxiety levels before and after the second and third TSST exposure, respectively, were almost identical. It may therefore be argued that subjects are in a high anxiety context during the first TSST session and in a low anxiety context when confronted with the second and third TSST exposure.

To the best of our knowledge, this is the first report documenting the relevance of context for psychoendocrine heritability estimates within a relatively confined time range. In general, the importance of environment and context is, of course, a well-known concept in genetics (e.g. see Ref. 15), and only recently a paper by Caspi et al. (38) showed that the effect of a genetic factor (a functional polymorphism in the promoter region of the serotonin transporter gene) on risk of major depression is markedly moderated by an environmental factor, namely the exposure to stressful life events.

In line with the above argument, one could speculate that a trait component of the endocrine stress response becomes more apparent with repeated stress exposures and that, as a consequence, higher contributions of genetic factors to variation in cortisol and ACTH responses to TSST exposure were estimated in the second and third session. This argument is supported by an earlier report on significant correlations between personality traits and cortisol stress responses that become apparent only after data aggregation across several TSST sessions (39). Thus, it could be argued that situational and psychological factors initially mask existing genetic influences and that novelty, unpredictability, and uncontrollability on the one hand and state-anxiety on the other hand represent such initially masking factors.

In line with the results obtained for the endocrine analytes, variations in HR responses to the first TSST exposure were not found to be influenced to a large degree by genetic factors (\( h^2 = 0.30 \)), but due to an increase of MZ intrapair correlations, higher heritabilities were observed in the second and third stress session. Contrary to the response reduction in HPA axis responses across sessions, a tendency toward increasing HR responses was observed from the first to the second exposure. Comparable dissociations between the reactivity of the HPA axis and the sympathetic nervous system to repeated psychosocial stress have been previously reported (32, 40). It is tempting to speculate that the tendency toward increasing HR responses from the first to the second TSST exposure is associated with the increasing heritabilities and may be explained by a trait component of the HR response becoming more apparent after repetition of the stressor. However, this explanation is rather unlikely because the increase of HR responses across sessions in our sample is not significant and can be explained to a large degree by the lower baseline levels before the first stress session. The question as to why increasing HR heritabilities are detected here has to remain open and should be addressed again in a larger sample.

One additional twin study investigated the question of a possible heritability of salivary cortisol responses to a single exposure to psychosocial stress (11). The influence of genetic factors on variation in salivary cortisol levels after the first TSST exposure in our sample (\( h^2 = 0.08 \)) is distinctly lower than the moderate heritability of \( h^2 = 0.32 \) these authors found for the same psychosocial stress protocol (TSST). For at least two reasons, we believe that these findings are conflicting only at first glance. First, these researchers included opposite-sex DZ twin pairs in their sample, which might be one reason for the higher heritabilities they found in the TSST and also in a CRH test (\( h^2 = 0.84 \)). Second, the sample size in this study (13 MZ, 11 DZ) is considerably smaller than the sample size in the study presented here. All other reports on the heritability of cortisol and ACTH responses to one single (pharmacological) HPA axis stimulation reveal low heritabilities, if any, and are thus in line with the present report (10, 12, 13).

Interestingly, our data suggest higher heritabilities of salivary and total cortisol under stimulated conditions, compared with basal and recovery values. Comparably, a recent
study revealed higher blood pressure heritabilities under mental stress conditions, compared with rest measurements (41). Using a structural equation modeling procedure, these researchers showed that the same genetic and environmental influences are expressed under stress and rest conditions. An amplified impact of genetic factors under stressful conditions in one or more of the systems involved in regulating HPA axis activity is a plausible assumption for our data as well. Furthermore, it is possible that additional genes are of importance under stress conditions. For example, glucocorticoid receptors (GRs) have only one tenth of the affinity of mineralocorticoid receptors, and increased binding to GRs occurs only under conditions of considerably elevated corticosteroid levels (for a review see Ref. 42). It is therefore plausible to speculate that an additional effect of genes associated with GR function can be observed under conditions of stimulated HPA axis activity that is not detectable under basal conditions. In line with this argument, two common polymorphisms of the GR gene were found to be associated with TSST responses in this sample (43). Whereas homozygous carriers of the G allele of a Bell restriction fragment length polymorphism, which is now identified as a C/G SNP in intron 2 (44, 45), display a significantly diminished salivary cortisol response to the stressor, carriers of the 363S allele of the N363S polymorphism of the GR gene (46) show significantly increased salivary cortisol responses. However, no genotype differences in prestress hormone levels could be detected for either polymorphism.

Although in the present study 58 twin pairs were recruited to participate in a relatively extensive investigation that included three visits in our laboratory, this sample size does not allow data analysis with genetic model-fitting techniques (e.g. Mx) (47). This is a problem that other twin studies with a comparably extensive design encounter as well. Thus, it is not very surprising that, although it would be desirable, at present no original study on the heritability of stimulated HPA axis activity has used this statistical methodology. Another limitation of this marker is the possibility of mathematically resulting heritabilities of 1, which cannot be interpreted as a genetically determined response. Rather, these findings indicate a high influence of genetic factors, and the focus of our argument is not the degree of heritability per se but rather the increase in heritabilities that clearly occurs. However, for at least two reasons, we feel confident that the employed method did not significantly bias our findings. First, the pattern of our results is rather consistent for salivary and total cortisol, ACTH, and HR. And second, a recent reanalysis of five comparable twin studies (9) revealed a heritability of 62% for basal cortisol secretion using model-fitting techniques, which is higher than the heritabilities each of these studies would have reported using the approach in the present study.

The data obtained in the present study have some clinical and methodological implications. First, the results of the present study point toward a considerable influence of heritable factors on HPA axis reactivity. Future studies investigating the sources of stimulated HPA axis variation should take this finding into account, and future twin and molecular genetic studies should aim to further elucidate this finding. Second, it seems important to note that twin studies relying on hormone responses to one single stress exposure may result in a distinct underestimation of the contribution of genetic factors to the total variance in cortisol, ACTH, and HR responses. Methodologically, this implies that heritabilities obtained in studies on HPA axis reactivity should be reevaluated in light of this finding, especially because some previous studies suggest an intr individually relatively stable pattern of HPA axis responses across various types of stressors (48, 49). Possibly these data also have consequences for the assessment of basal hormone levels because situational factors may impact predominantly on the first day of basal hormone assessment as well. Finally, it should be noted that repeated exposure to identical moderate stressors is not limited to laboratory studies but occurs on a regular basis in everyday life. The result of this study could imply that heritable factors might be less important with regard to a person’s stress response in a new, uncontrollable, unpredictable, and anxiety-evoking situational context. Instead, they would rather be relevant in response to repeated or chronic stress and, consequently, in HPA axis dysregulations and associated HPA axis related diseases.

Taken together, these results indicate increasing heritabilities for salivary cortisol, total cortisol, ACTH, and HR responses after repeated exposure to the same psychosocial stressor, which might be explained by a change in the environmental context in which the stressor occurs. Furthermore, higher salivary and total cortisol heritabilities were observed under conditions of mental stress, which could either be due to an amplification of the same genetic factors or an impact of additional genes under stress conditions.

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