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Nonsynonymous *HTR2C* polymorphism predicts cortisol response to psychosocial stress II: Evidence from two samples

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ABSTRACT

The 5-HT$_{2C}$ receptor is the primary serotonin receptor located in the corticotrophin releasing hormone (CRH) neurons of the hypothalamus. These neurons initiate the signaling cascade that culminates in cortisol release. Therefore, genetic variation in the 5-HT$_{2C}$ receptor gene (*HTR2C*) is a prime candidate for affecting cortisol reactivity to stress. Accordingly, we examined the association of a nonsynonymous polymorphism (Cys23Ser; rs6318) in *HTR2C* with stress reactivity in two Trier Social Stress Tests conducted at separate sites. In both Study 1 (N = 128) and Study 2 (N = 185), Cys23 homozygous females and hemizygous males had greater cortisol reactivity. There was no relation between this polymorphism and self-reported affective response (Studies 1 and 2) or cardiovascular reactivity (Study 2). Additionally, the short/short genotype of a polymorphism (5-HTTLPR) in the serotonin transporter gene was associated with greater cortisol reactivity in Study 1 as well as in Study 2 (previously reported). The Cys23Ser polymorphism and the 5-HTTLPR were independently associated with cortisol reactivity in both studies. These findings emphasize the important role of genetic variation in the serotonin system on regulating cortisol reactivity to social evaluative stress. Comparison of the present associations with those of prior studies underscores the likely importance of situational and psychological factors in determining the direction and magnitude of the association between genotype and phenotype.

1. Introduction

In daily life, people regularly encounter stressful events. When such events are uncontrollable, painful, or threatening, they can elicit activation of corticotrophin releasing hormone (CRH) neurons in the hypothalamus. This initiates a signaling cascade that culminates with the release of cortisol from the adrenal cortex. A key regulator of reactivity within this hypothalamic-pituitary-adrenal (HPA) axis is the serotonin (5-HT) system (Chaouloff et al., 1999). Serotonin neurons directly innervate CRH neurons in the hypothalamus (Liposits et al., 1987) as well as the limbic and paralimbic areas (Way et al., 2007) that project to the hypothalamus. Based on rodent data, the most prevalent serotonin receptor in CRH neurons is the 5-HT$_{2C}$ receptor (Heisler et al., 2007). Deletion of this receptor nearly eliminates the increased release of CRH from the hypothalamus following serotonin release. In humans, pharmacological challenge with an agonist acting on the 5-HT$_{2C}$ receptor leads to increases in cortisol release (Kahn et al., 1990; Seibyl et al., 1991). Therefore, the 5-HT$_{2C}$ receptor is a critical driver of HPA axis reactivity.

In the human 5-HT$_{2C}$ receptor gene (*HTR2C*), there is a polymorphism (rs6318) that leads to a substitution of a serine for a cysteine (Cys23Ser) in the coding region (Lappalainen et al., 1995). Although the precise mechanism by which this polymorphism affects cellular signaling has not been conclusively determined (Fentress et al., 2005; Okada et al., 2004; Walstab et al., 2011), accumulating evidence suggests that the Cys23Ser polymorphism impacts reactivity to psychological stress. In women homozygous for the Ser23 allele, high levels of exposure to stressful life events have been associated with higher depressive symptoms than in women carrying the Cys23 allele exposed to similar levels of life stressors (Brummett

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et al., 2014b). In a large sample of individuals with coronary artery disease, women homozygous for the Ser23 allele had higher risk of myocardial infarction and all-cause mortality than those carrying the Cys23 allele (Brummett et al., 2013). Males with the Ser23 allele (hemizygous) showed the same risk as women homozygous for the Ser23 allele. Thus, the Ser23 allele appears to be associated with multiple stress-related health outcomes.

Because the HPA axis shows dysregulation in certain subtypes of depression (Stetler and Miller, 2011) and in cardiovascular disease (Hamer et al., 2012; Pereg et al., 2011), cortisol may be a common contributor to these mental and physical health outcomes. In fact, the Ser23 allele was associated with greater cortisol release during an emotional recall task in a study of men (Brummett et al., 2012) and in a replication sample of men and women (Brummett et al., 2014a). This is consistent with findings showing that Ser23 carriers exhibit greater central dopamine release during a pain stressor (Mickey et al., 2012) known to increase cortisol levels (Peciña et al., 2013). In both the emotional recall task and the pain tasks, the Ser23 allele was associated with greater emotional reactivity. This suggests that the Ser23 allele is a risk factor for stress-related mental and physical health outcomes.

A more commonly used laboratory measure to assess cortisol reactivity is the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), which involves delivering a speech and performing mental arithmetic in front of an evaluative audience. Such social evaluative threat reliably impacts HPA axis activation and is a common experience in modern societies (Lehman et al., 2015; Smith et al., 2012). The goal of the present studies was to determine if the Cys23Ser polymorphism affects cortisol response in the TSST, an analogue to daily life social threat. To build confidence in the results, we tested this association with two study designs at separate sites. In Study 1, we examined the association of CysSer23 with TSST cortisol response in both working adults and college students in a correlational fashion. In Study 2, we sought to replicate the observed relation with an experimental TSST design in a mixed working adult and college student sample. Based on prior work (Brummett et al., 2014a, 2012), we anticipated that the Ser23 allele would be associated with greater cortisol reactivity.

In addition to examining the role of variation in the *HTR2C* gene in moderating stress reactivity, we also sought to replicate the effects of a polymorphism (5-HTTLPR) in the promoter region of the serotonin transporter gene. A recent *meta*-analysis supports a role for the 5-HTTLPR short/short genotype in greater cortisol reactivity to stress (Miller et al., 2013), but there was heterogeneity in the results. For example, one study found that the long allele was associated with greater cortisol reactivity (Mueller et al., 2011). Therefore, to expand the knowledge base regarding the relationship between the 5-HTTLPR and stress reactivity, we examined this polymorphism's contribution to TSST-evoked cortisol response both independently and in the context of 5-HT_{2C} effects. To examine the specificity of these genetic associations to HPA axis functioning, we also examined their relations to cardiovascular and subjective negative emotional responses.

2. Methods: Study 1

2.1. Participants

Participants were 149 employees (n = 81; sample 1) and students (n = 68; sample 2) at Virginia Commonwealth University (VCU), a large public institution, and were recruited through poster and e-mail advertisements. Inclusion of two populations, college students and working adults, was designed to enhance the generalizability of the research findings across major sociodemographic groups. Inclusion criteria were age (> 18 years) and ability to read and write in English. Exclusion criteria were: 1) existing health conditions (e.g., autoimmune disorders) or health habits (e.g., regular cigarette, illicit drug, oral contraceptive use) that could affect stress responsiveness or increase health risks during the TSST; 2) health conditions (e.g., Cushing's disease, high blood pressure, psychiatric illness) or drug use (e.g., marijuana, tobacco) that could affect cortisol levels. Participants were asked to refrain from strenuous exercise, alcohol consumption, and smoking on the session day, and to refrain from consuming dairy products, caffeine, or eating < 1 h before the session (c. f., Gruenewald et al., 2004). Inclusion and exclusion criteria were checked at screening and upon entry into the TSST session. Of the initial participants, 5 were removed before analysis for procedural errors, and 11 for non-compliance in the self-report or TSST portion of the study. Three participants were removed for current psychiatric illness, and 1 was removed for English language comprehension difficulty. This left N = 129 participants for analysis (n = 62; sample 1; n = 67; sample 2).

Sample 1 (employees) was 71% female, with an average age of 38.34 years (SD = 11.47). Most were Caucasian (n = 42; 67.7%); the balance were Black or African American (n = 15; 24.2%); Asian (n = 4; 6.5%) and Hispanic or Latino(a) (n = 1; 1.6%). Sample 2 (students) was 61% female, with an average age of 21.09 years (SD = 3.43). This sample was more racially and ethnically diverse. Caucasians comprised 33.3% (n = 22), Black or African American participants comprised 30.3% (n = 20), and Asian individuals comprised 22.7% (n = 15) of the sample. The balance were Hispanic or Latino(a) (n = 3; 4.6%), mixed race (n = 1; 1.5%), another racial/ethnic group (n = 3; 4.6%), or undeclared (n = 2; 3.0%). Participants earned $100 for study completion. All procedures were approved by the Institutional Review Board at VCU.

2.2. Procedure

The testing session (2 h in duration) was conducted in a study-dedicated laboratory suite between 1 and 6 pm to control for diurnal cortisol variation. After an introduction to the study and receipt of written informed consent, baseline saliva for cortisol assay was collected, and blood for DNA extraction was drawn from the non-dominant arm by a trained phlebotomist via indwelling catheter with a butterfly needle and saline-lock access.[1] A local anesthetic cream was applied to the venipuncture site to minimize pain. Stress-relevant measures of current psychological state were also completed (see Section 2.3 below). All saliva and blood samples were processed immediately post-session and cryopreserved until analysis, after which any remaining samples were destroyed.

Following baseline data collection, participants began the TSST, which followed standard procedures (Kirschbaum et al., 1993). Participants spent 5 min mentally preparing a five-minute public speech before delivering it to a panel of two critical peer evaluators. For sample 1, the speech was to address: "Why you would be a good candidate for a job in which you would have to work effectively with university staff to come up with solutions to problems typically faced by employees in your department or unit." For sample 2, participants were asked to explain "Why you would be a good candidate for a job as an administrative assistant in the university Psychology Department." After this speech preparation period, two additional, brief self-report measures not discussed here (achievement goals and primary appraisal) were completed.

The experimenter waited in an adjoining control room during speech preparation, and subsequently, two public speech "evaluators" (trained confederates) entered the participant room and

[1] For other study purposes, two additional blood draws were made: 45 min and 85 min after TSST onset (data not reported here).

sat at a desk directly in front of the participant. One of the speech evaluators indicated to the participant the 4 recording video cameras placed around the room, started a 5-min timer, and said, "Please begin your speech; you have five minutes." If the participant inquired about the time remaining or stopped their speech before 5 min, the evaluators asked him/her to continue speaking. At the end of the speech, the participant was instructed to perform, as quickly and accurately as possible, a mathematical subtraction task before the same critical evaluators for 5 min. The evaluators took notes during the tasks to enhance experimental realism. At standardized intervals over the next 45 min, saliva samples and affect measures were collected while the participant rested (see Section 2.3 below). The participant was then debriefed, thanked, and dismissed.

2.3. Measures

2.3.1. Salivary cortisol

Saliva samples were collected five times during the 90-min lab session to assess peak cortisol reactivity and recovery (Dickerson and Kemeny, 2004). Samples were collected via 2-min sublingual placement of synthetic Salivettes (Salimetrics, State College, PA) at baseline, immediately after the speech task (10 min from task onset), and 10, 20, and 35 min after the tasks (20, 30, and 45 min from task onset). After the session, samples were stored at −20 °C until batch processed. Samples were then thawed and centrifuged for 15 min at 1500*g* at 10 °C. Cortisol was assayed using the Salimetrics competitive immunoassay method. Inter-assay coefficient of variation (CV) was 6.69—6.88%, intra-assay CV was 3.88—7.12%, and the sensitivity was < 0.007 μg/dL.

2.3.2. Negative affectivity

The NA portion of the Positive and Negative Affect Schedule (PANAS; Watson et al., 1988) assessed negative affectivity "currently" at baseline ($\alpha = 0.73$), and at 10, 20, and 35 min after the TSST (20, 30, and 45 min from task onset).

2.3.3. Anxiety

The 9-item Profile of Mood States anxiety subscale (POMS; McNair et al., 1971) assessed anxiety "currently" (baseline $\alpha = 0.80$) on the same assessment schedule as with NA.

2.3.4. Genotyping

Samples were genotyped for the rs6318 polymorphism using a Taqman SNP Genotyping Assay Kit (C___2270166_10; Thermofisher) according to the manufacturer's instructions. Polymerase chain reactions were performed using 20 μL reaction volumes in 96-well plates with 25 ng of DNA. End point reads of fluorescence levels were obtained with an Applied Biosystems Incorporated (ABI) 7300HT Sequence Detection System. For quality control, 20% of genotypes were rerun and showed perfect concordance. All samples were successfully genotyped (DNA for one subject was exhausted after assaying the 5-HTTLPR). Haploview (version 4.2; (Barrett et al., 2005)) was used to calculate Hardy–Weinberg Equilibrium values using the exact test (Wigginton et al., 2005). Because the Cys23Ser polymorphism is on the X-chromosome, this test was conducted on female participants only (analysis of the main dependent measures included both males and females; see Section 2.4.1). The distribution of genotypes did not deviate from Hardy-Weinberg equilibrium ($p = 0.19$).

The 5-HTTLPR was genotyped using primers from Gelernter et al. (1997) and a protocol modified from Heils et al. (1996) by Anchordoquy et al. (2003). The forward primer was 5′-ATGCCAGCACCTAACCCCTAATGT-3′ (labeled with 6-carboxyfluorescein fluorophore) and the reverse primer was 5′GGACCGCAAGGTGGGCGGGA-3′. These primers yielded amplicons of 376 bp (short) or 419 bp (long). Polymerase chain reaction (PCR) was performed in a total volume of 20 μL, containing 100 ng of DNA; 10 nM of each primer; 1x buffer; 2 mM MgCl2; 10% DMSO (v/v); 2U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA); 200 μM of dATP, dCTP, and dTTP; 100 μM of dGTP; and 7-deaza-2′-dGTP. Cycling conditions consisted of (1) an initial 12 min denaturation at 94 °C; (2) touchdown from 65 °C–55 °C decreasing by 0.5 °C per cycle with denaturation for 30 s at 94 °C, varied annealing temperatures consisting of 30 s at 66 °C (2 cycles), then 65 °C (3 cycles), then 64 °C (3 cycles), followed by hybridization for 1 min at 72 °C; (3) 35 cycles with an annealing temperature of 63 °C and the same denaturation and hybridization parameters; and (4) a final extension for 20 min at 72 °C. The PCR products were electrophoresed on an ABI 3730 DNA analyzer (Applied Biosystems) with Genescan LIZ500 size standard (Applied Biosystems). Data collection and analysis used GeneScan and Genemapper software (Applied Biosystems). For quality control, 13% of genotypes were rerun and showed perfect concordance. Two samples were heterozygous for an extra-long Long allele (81 bp) and were omitted from analyses due to potential differential effects on gene expression (Vijayendran et al., 2012). One sample could not be genotyped. Because of the racial/ethnic heterogeneity of the entire sample, there was a deviation from Hardy Weinberg equilibrium ($p = 0.012$). However, there was no deviation within the largest ethnic group (Caucasian; $p = 0.35$), indicating that there was not allele dropout (Kaiser et al., 2002; Yonan et al., 2006).

2.4. Genetic and statistical analyses

2.4.1. Cys23Ser and 5-HTTLPR allele coding

The *HTR2C* gene is located on the X-chromosome. Because males only have one X chromosome, they can only have one rs6318 allele: either Ser23 or Cys23. Females, on the other hand, have two X chromosomes, meaning that they have two alleles at this locus and can therefore have three genotypes (Cys23/Cys23, Cys23/Ser23, or Ser23/Ser23), just like genes located on other chromosomes. Because of these differences in allele number between males and females for many genes on the X Chromosome processes such as inactivation of one allele on the X-chromosome of females (Carrel and Willard, 2005) or upregulation of expression on the male X chromosome (Johnston et al., 2008) appear to compensate for the difference in allele numbers in most X-linked genes. This appears to be the case for the *HTR2C* gene, because there is little difference in methylation or gene expression between males and females (Hernando-Herraez et al., 2013). Therefore, we analyzed males and females together. Because of the small number of Ser23 hemizygotes (males) or homozygotes (females), we grouped the heterozygotes (Ser/Cys) with the Ser/Ser group, as has been done previously (Mickey et al., 2012) to create a Ser23 carrier category. Thus, this polymorphism was coded as 0 = Cys23/Cys23 (females) or Cys23 (males); 1 = Ser23 carrier (females: Cys23/Ser23, Ser23/Ser23; males: Ser23). In the supplementary online material, we present the data for each genotype separately for qualitative comparisons. The 5-HTTLPR polymorphism was coded as 0 = short/short; 1 = short/long; and 2 = long/long, as in prior work (Way and Taylor, 2010). Alternative analyses accounting for the rs25531 polymorphism are presented in the supplementary online material.

2.4.2. Statistical analyses

Analyses of the full repeated cortisol and affect outcomes were conducted using restricted maximum likelihood (REML) mixed models. Polymorphism differences were tested as main effects and in interaction with time (the latter as linear and curvilin-

ear slopes). Participant sex, age, race, number of previous night sleep hours, past week dietary and exercise variables, and session time were covaried in preliminary analyses. Given the racial/ethnic distribution of the two samples, the race variable was coded as Black/African American = 1, all others = 0; and Caucasian/European American = 1, all others = 0. These two variables were entered as covariates in all models to control for population stratification artifacts. Due to positive skewness, cortisol values were natural log-transformed and state PANAS NA scores were square-root transformed. Choice of the most appropriate variance-covariance structure (unstructured, compound symmetry, toeplitz, variance components) and within-person error variance-covariance structure (first-order autoregressive) was determined through chi-square tests comparing the −2 restricted log likelihood model fit indices for each outcome. A variance components structure without autoregression was used in all models. The MIXED procedure in SAS (v 9.4) was used to estimate all REML mixed models. Across models, denominator degrees of freedom varied slightly due to data missingness.

3. Results: Study 1

3.1. Preliminary analyses

We first tested whether cortisol levels and affective responses were predicted by TSST phase (saliva collection time points coded 0-4) while co-varying samples (adults, students), demographic, sleep, dietary, exercise, and session start time variables. As expected, a mixed model revealed a significant time × time (time^2), or curvilinear change (rise and fall) in cortisol [$b = -0.111$, $SE(b) = 0.007$, $t(513) = -14.96$, $p < 0.001$]. Among the participant covariates, females showed higher cortisol levels across time [$b = 0.323$, $SE(b) = 0.098$, $t(122) = 3.30$, $p = 0.001$]. Caucasians also showed higher cortisol levels across time [$b = 0.331$, $SE(b) = 0.124$, $t(122) = 2.68$, $p = 0.009$], as did African Americans [$b = 0.573$, $SE(b) = 0.132$, $t(122) = 4.34$, $p < 0.0001$]. In addition, later session times predicted lower cortisol responses [$b = -0.140$, $SE(b) = 0.061$, $t(122) = -2.28$, $p = 0.024$] and fewer days eating breakfast over the past 7 days predicted higher cortisol [$b = -0.061$, $SE(b) = 0.021$, $t(122) = -2.96$, $p = 0.004$]. Sample (1 and 2) did not predict cortisol levels over time [$b = -0.021$, $SE(b) = 0.108$, $t(122) = -0.19$, $p = 0.85$]. The significant covariates were included in subsequent analyses of cortisol response.

PANAS NA showed a curvilinear rise and fall over time [$b = -1.493$, $SE(b) = 0.072$, $t(255) = -20.86$, $p < 0.0001$] as did POMS anxiety [$b = -1.334$, $SE(b) = 0.061$, $t(256) = -21.97$, $p < 0.0001$]. None of the control variables (sample, sex, race, session time, and past week breakfasts) predicted NA ($ps > 0.06$). Earlier session times predicted higher anxiety [$b = -0.146$, $SE(b) = 0.063$, $t(123) = -2.32$, $p = 0.02$], but the other control variables did not ($ps > 0.29$).

3.2. Primary analyses: the Cys23Ser polymorphism

The primary analyses examined whether the Cys23Ser polymorphism moderated the cortisol and affect response curves observed in the TSST. The rarer Ser23 allele carriers were present in 29.7% of the combined sample ($n = 38$; Table 1). In a mixed model predicting salivary cortisol response, a Ser23 × time^2 interaction was observed [$b = -0.051$, $SE(b) = 0.016$, $t(487) = -3.27$, $p = 0.001$], such that Cys23 participants (i.e., non-Ser23 carriers) showed elevated cortisol response to the TSST (see Fig. 1). This effect was in the opposite direction to that expected. There were no differences in baseline cortisol levels ($t(125) = 1.31$, $p = 0.19$).

The curvilinear patterns of NA and anxiety did not differ according to the Cys23Ser polymorphism (NA: [$b = 0.291$, $SE(b) = 0.160$, $t(241) = 1.82$, $p = 0.07$]; anxiety: [$b = 0.228$, $SE(b) = 0.133$, $t(242) = 1.71$, $p = 0.09$), although the Ser23 allele carriers reported marginally higher NA and anxiety. Session time did not predict NA [$b = -0.084$, $SE(b) = 0.071$, $t(116) = -1.18$, $p = 0.24$] but did predict anxiety [$b = -0.144$, $SE(b) = 0.065$, $t(116) = -2.22$, $p = 0.03$]. Neither sex nor the race variables predicted NA ($ps > 0.08$) or anxiety ($ps > 0.51$), nor did number of breakfasts over the past week ($ps > 0.45$).

Table 1
Distribution of genotypes across Study 1 and Study 2.

Cys23Ser Polymorphism				
		Cys23		Ser23
Males	Study 1	36		8
	Study 2 Evaluation Condition	44		1
	Study 2 Control Condition	22		2
		Cys23/Cys23	Cys23/Ser23	Ser23/Ser23
Females	Study 1	54	24	6
	Study 2 Evaluation Condition	66	8	0
	Study 2 Control Condition	36	6	0
5-HTTLPR Polymorphism				
		Short/Short	Short/Long	Long/Long
	Study 1	28	45	53
	Study 2 Evaluation Condition	41	53	25
	Study 2 Control Condition	27	27	11

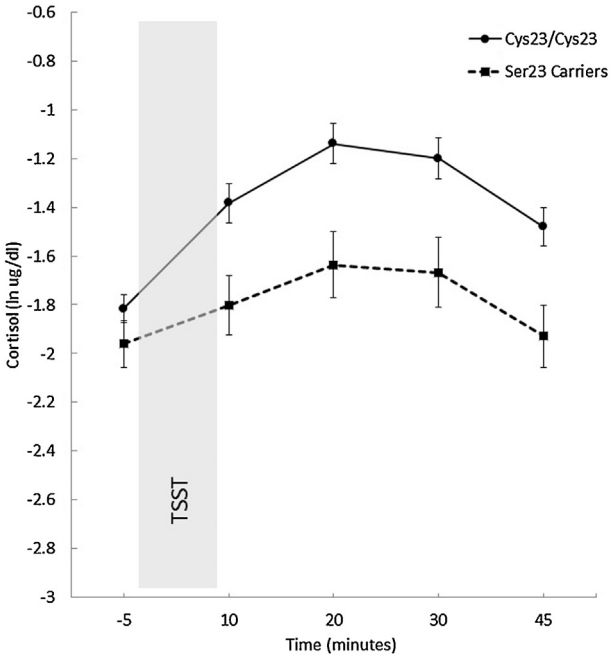

Fig. 1. Mean (±SEM) cortisol response to the Trier Social Stress Test as a function of the Cys23Ser polymorphism (Study 1). Cys23/Cys23 denotes homozygous females and hemizygous males for the Cys23 allele. Ser23 carrier denotes heterozygous females as well as homozygous females and hemizygous males. Ordinate values are the natural logarithm (Ln) of the raw cortisol values. The gray vertical bar denotes the speech and mental arithmetic portion of the TSST.

3.3. Primary analyses: the 5-HTTLPR polymorphism

Replicating prior work, the 5-HTTLPR × time^2 interaction was significant in a mixed model [short/short *vs* long/long; $b = -0.062$, $SE(b) = 0.019$, $t(467) = -3.30$, $p = 0.001$] and [short/long vs long/long; $b = 0.032$, $SE(b) = 0.016$, $t(467) = 1.96$, $p = 0.05$], such that the short/short genotype predicted the highest cortisol reactivity.

We then tested whether the Ser23 allelle would continue to moderate the effect of evaluation on cortisol responses when

terms representing the main effect of 5-HTTLPR and its interactions with linear and quadratic time were added to the mixed model. The Ser23 × time^2 interaction remained significant in this model [$b = -0.035$, $SE(b) = 0.016$, $t(465) = -2.19$, $p = 0.029$] as did the 5-HTTLPR, particularly the short/short vs long/long contrast [$b = -0.055$, $SE(b) = 0.019$, $t(465) = -2.85$, $p = 0.005$]. The short/long *vs* long/long contrast became nonsignificant in this model [$b = 0.030$, $SE(b) = 0.016$, $t(465) = 1.89$, $p = 0.06$]. Thus, both the 5-HTTLPR and the CysSer23 polymorphism independently predicted stress-induced cortisol responses over time. In the prediction of NA, the 5-HTTLPR did not interact with linear time ($ps > 0.21$) or quadratic time [short/short *vs* long/long; $b = -0.226$, $SE(b) = 0.198$, $t(227) = -1.14$, $p = 0.25$] and [short/long *vs* long/long; $b = 0.162$, $SE(b) = 0.167$, $t(227) = 0.97$, $p = 0.33$]. Similarly the 5-HTTLPR did not interact with linear time ($ps > 0.24$) or quadratic time [short/short *vs* long/long; $b = -0.159$, $SE(b) = 0.168$, $t(229) = -0.95$, $p = 0.34$] and [short/long vs long/long; $b = 0.031$, $SE(b) = 0.142$, $t(229) = 0.22$, $p = 0.83$] to predict anxiety.

4. Study 2

Study 2 was designed to replicate and extend the results of Study 1 with the use of an experimental TSST design. This permitted a test of whether the association between Cys23Ser and cortisol response observed in Study 1 was specific to social evaluative threat. The participants and procedures were the same as in prior published work (Way and Taylor, 2011, 2010), so the study methods are only briefly summarized here.

5. Methods and materials: Study 2

5.1. Participants

Participants were students or employees at UCLA, a large public university. The sample (N = 185) was 63% female, ranged in age from 18 to 35 years (M = 21.3), and had the following racial/ethnic composition: 38% Asian-American, 22% European-American, 16% Latino, 23% mixed race/ethnicity, and 2% African-American. Participants received $120 for completing the study.

5.2. Procedures: Trier Social Stress Test

All procedures were performed in the mid-to-late afternoon to control for diurnal variation in cortisol. Participants individually completed the TSST using procedures similar to those used in Study 1, except that each was randomized into an evaluative condition or a control condition for which the audience was not present. Further details about the conditions are described in the supplementary material. Participants delivered a speech on why they were the best person for an administrative assistant position. Five cortisol samples were taken: 60 and 30 min before the TSST (averaged together to form a baseline) and then 20, 40, and 75 min after onset of TSST instructions. Participants completed the PANAS (Watson et al., 1988) 5 min before being given the TSST instructions and 15 min after the conclusion of the TSST.

5.3. Measures

5.3.1. Salivary cortisol

Saliva was collected via passive drool into a 2.0 mL Corning cryovial (Corning, NY), immediately iced, and at the end of each session transferred to a −20 °C storage freezer. The radioimmunoassay was run in duplicate with 25 μL samples with the High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics, State College, PA). The inter- and intra-assay coefficients of variation were 6.1% and 5.0% respectively.

5.3.2. Cardiovascular responses

Systolic and diastolic blood pressure (SBP and DBP, respectively) and heart rate (HR) were assessed via a Critikon Dinamap Vital Signs Monitor Model 1846SX (Tampa, FL). These assessments were performed at 7 time points: at baseline and then at 5 min intervals corresponding to the speech instructions, speech preparation, speech delivery, mental arithmetic (result averaged with speech task), and at two recovery points.

5.3.3. Negative affect

The NA portion of the PANAS was used to assess state negative affect (baseline $\alpha = 0.85$)

5.3.4. Genotyping

DNA was collected from saliva with Oragene kits (DNA Genotek, Ottawa, Ontario, Canada) and extracted according to manufacturer recommendations. Genotyping of rs6318 was performed as in Study 1. All samples were successfully genotyped. For quality control, 28% were rerun and showed perfect correspondence. As with Study 1, the genotyping quality check using Hardy-Weinberg equilibrium calculation was done on females only and did not deviate from equilibrium ($p = 0.52$). Analyses of the dependent measures included both males and females. The genotyping procedure for the 5-HTTLPR is described in the supplementary online material. One participant could not be genotyped for the 5-HTTLPR.

5.4. Statistical analyses

Analyses were conducted as in Study 1, with the following exceptions. The racial/ethnic distribution of this sample indicated that this variable be appropriately dummy coded as Asian-American = 1, all others = 0; and Caucasian/European-American = 1, all others = 0. SBP, DBP, and HR reactivity were analyzed with REML mixed models using the 7 repeated measures. Preliminary analysis showed that for both the cortisol and cardiovascular outcomes, the most appropriate variance-covariance structure was unstructured, with a first-order autoregressive within-person error variance-covariance structure. The MIXED procedure in SAS (v 9.4) was used to estimate all REML mixed models. As in Study 1, denominator degrees of freedom varied slightly across models due to data missingness. Because NA was collected only twice (pre- and post-TSST) least squares multiple regression analyses were performed on this outcome, using the REG procedure in SAS (v 9.4).

6. Results: Study 2

6.1. Preliminary analyses

As in Study 1, we first tested whether cortisol levels were predicted by TSST phase, with saliva collection time points coded 0-3. In this and all following REML mixed models, sex and ethnicity were controlled. A mixed model revealed a significant time^2, or curvilinear change (rise and fall) in cortisol [$b = -0.252$, $SE(b) = 0.016$, $t(545) = -15.27$, $p < 0.0001$]. A condition × time^2 interaction was also found in cortisol [$b = 0.123$, $SE(b) = 0.027$, $t(545) = 4.55$, $p < 0.0001$] such that the evaluative TSST conditions produced higher cortisol levels than the control condition. Neither sex [$b = 0.084$, $SE(b) = 0.092$, $t(179) = 0.92$, $p = 0.36$] nor race (Asian: $b = -0.074$, $SE(b) = 0.098$, $t(179) = -0.75$, $p = 0.45$; European: $b = 0.086$, $SE(b) = 0.117$, $t(179) = 0.74$, $p = 0.46$) predicted average cortisol levels across time. These variables were also nonsignificant predictors in interaction with linear and quadratic time ($ps > 0.05$). The evaluative TSST conditions did not produce greater

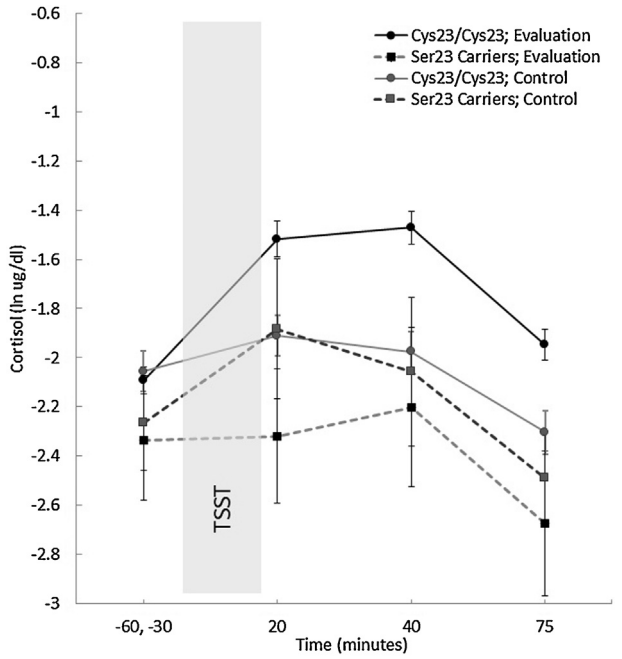

Fig. 2. Mean (±SEM) cortisol response to the Trier Social Stress Test as a function of the Cys23Ser polymorphism in the evaluative audience and no audience (control) conditions (Study 2). Ordinate values are the natural logarithm (Ln) of the raw cortisol values. The gray vertical bar denotes the speech and mental arithmetic portion of the TSST.

negative affect than the control condition after controlling for baseline levels ($p = 0.90$).

6.2. Primary analyses: the Cys23Ser polymorphism

The primary analyses examined whether the Cys23Ser polymorphism moderated the effect of experimental condition on TSST cortisol response. Ser23 allele carriers were 9.3% of the sample ($n = 17$; Table 1). In this mixed model, a Cys23Ser × condition × time^2 interaction was observed [$b = 0.222$, $SE(b) = 0.091$, $t(541) = 2.44$, $p = 0.015$], such that participants without the Ser23 allele (i.e., Cys/Cys females and Cys males) showed higher cortisol response in the evaluative conditions of the TSST (see Fig. 2). In this model the condition × time^2 interaction dropped to nonsignificance [$b = -0.077$, $SE(b) = 0.086$, $t(541) = -0.89$, $p = 0.38$]. According to an ANOVA model with baseline cortisol levels as the dependent measure, there was no main effect of genotype ($F(1, 178) = 0.06$, $p = 0.81$), condition ($F(1, 178) = 1.79$, $p = 0.18$), or their interaction ($F(1, 178) = 0.03$, $p = 0.87$). In a multiple regression analysis that controlled for pre-TSST NA, the Cys23Ser polymorphism did not predict post-TSST NA, neither as a main effect nor interaction with condition and/or pre-TSST NA ($ps > 0.38$); neither the sex nor the race variables predicted NA ($ps > 0.45$).

6.3. Primary analyses: the 5-HTTLPR polymorphism

Paralleling Study 1, we tested whether the Cys23Ser polymorphism would continue to moderate the effect of evaluation on cortisol responses when terms representing the main effect of 5-HTTLPR and its full complement of two-way and three-way interactions with condition and time were added to the mixed model. As expected, the 5-HTTLPR × condition × time^2 interaction was significant—specifically the short/short vs long/long comparison [$b = 0.159$, $SE(b) = 0.078$, $t(529) = 2.05$, $p = 0.041$; short/long vs long/long: $b = 0.057$, $SE(b) = 0.077$, $t(529) = 0.74$, $p = 0.46$]. The Cys23Ser × condition x time^2 interaction remained significant in this model [$b = 0.214$, $SE(b) = 0.092$, $t(529) = 2.33$, $p = 0.020$]. Thus, conceptually replicating Study 1, the Cys23Ser polymorphism provided incremental value to the prediction of TSST cortisol responses over time.[2] In a multiple regression model of the Cys23Ser polymorphism that added the 5-HTTLPR main effect and interactions with condition and pre-TSST NA to the prediction of post-TSST NA, none of these terms predicted post-TSST NA ($ps > 0.14$).

Finally, we examined whether the Cys23Ser polymorphism moderated the effect of evaluation on cardiovascular responses (SBP, DBP, and HR), given prior associations of the Cys23Ser polymorphism with myocardial infarction (Brummett et al., 2013). MLM analyses showed strong curvilinear change in these outcomes [SBP: $b = -3.162$, $SE(b) = 0.384$, $t(545) = -8.23$, $p < 0.0001$; DBP: $b = -2.844$, $SE(b) = 0.257$, $t(545) = -11.08$, $p < 0.0001$; HR: $b = -3.187$, $SE(b) = 0.328$, $t(544) = -9.72$, $p < 0.0001$]. Only for HR did evaluation condition moderate this change [$b = 1.125$, $SE(b) = 0.539$, $t(544) = 2.09$, $p = 0.037$; other $ps > 0.29$]. No Ser23 × condition × time^2 interaction was found for the three cardiovascular outcomes ($ps > 0.22$).

7. General discussion

In two separate studies with three independent samples, we found that the Cys23 allele of the Cys23Ser 5-HT$_{2C}$ polymorphism was associated with greater cortisol reactivity in a social evaluative stressor task (TSST). In addition, the short/short genotype of the 5-HTTLPR was also associated with greater cortisol reactivity in Study 1 as well as in Study 2 (reported in Way and Taylor (2010)). The effects of each polymorphism were independent, in that each contributed significantly to the prediction of cortisol reactivity.

These results provide further support for a role of genetic variation in the serotonin system impacting cortisol reactivity to stress. However, the direction of the 5-HT$_{2C}$ polymorphism effect was opposite to that hypothesized based on prior literature. The genotype (Cys23 homozygous females and hemizygous males) associated with the greatest cortisol reactivity in the present studies was previously associated with *lower* cortisol response in two previous laboratory studies using an emotion recall paradigm (Brummett et al., 2014a, 2012) as well as lower risk for the depressogenic effects of stress in a large epidemiological sample (Brummett et al., 2014b). The present and prior findings seem to be contradictory. The most obvious potential explanation for these differential effects is the coding of genotypes across studies. In the two prior laboratory studies using an emotional recall paradigm to elicit cortisol response (Brummett et al., 2014a, 2012), there were no heterozygotes. Thus, the effect was driven by the comparison of the Cys23 genotype (male hemizygotes and female homozygotes) with the Ser23 genotype (male hemizygotes and a female homozygote). In the studies presented here, such a comparison would have been statistically unreliable with so few Ser23 homozygous females and hemizygous males ($n = 14$ in Study 1; $n = 3$ in Study 2). Nonetheless, inspection of means (see supplementary material) indicates that this latter group of individuals showed the lowest cortisol reactivity to the TSST, not the highest reactivity as would be expected based on prior work by Brummett et al. (2014a, 2012). Thus, the coding of genotypes cannot account for the discrepant results. What else could account for the differences in the direction of effect between the studies presented here and prior work? Below, we discuss potential psychological and neurochemical explanations for this effect.

[2] In both Studies 1 and 2, the cortisol results were very similar, and remained significant where indicated, when the Ser23 hemizygotes (males) and homozygotes (females) were removed from analyses ($n = 10$ and $n = 3$ in Studies 1 and 2, respectively).

7.1. Molecular mechanisms

One potential explanation for the contrasting 5-HT_{2C} effects is at the genetic level. *In vitro* research has suggested that the Ser23 allele is hypofunctional, requiring higher concentrations of serotonin to elicit the same response as the Cys23 allele (Okada et al., 2004). This would be consistent with the reduced cortisol reactivity seen here in individuals with the Ser23 allele. However, this polymorphism has also been found to have opposite effects (Walstab et al., 2011) or null effects (Fentress et al., 2005; Jahnsen and Uhlén, 2012) on receptor function. Until these molecular mechanisms are clarified, it is difficult to account for the differential allelic associations with cortisol reactivity at the molecular level.

Another possibility is that the Cys23Ser polymorphism is acting as a tag SNP in linkage disequilibrium with a nearby SNP that is functional. This could explain the different associations across studies if this unknown functional SNP were coupled with the Cys23 allele in the studies reported here and the Ser23 allele in the other studies showing the opposite effect (Brummett et al., 2014a, 2012). However, this seems unlikely because race/ethnicity is a main contributor to differences in linkage disequilibrium across individuals (Slatkin, 2008) and the two studies reported here had very different ethnic distributions, but the same association between Cys23Ser and cortisol reactivity.

This differential relation between Cys23Ser alleles and stress reactivity between the TSST and emotion recall paradigms is not limited to the Cys23Ser polymorphism, but also applies to the 5-HTTLPR. In addition to the effects of the 5-HTTLPR short/short genotype on cortisol response, we previously reported that the short/short genotype was associated with greater cardiovascular reactivity in the sample from the present Study 2 (Way and Taylor, 2011). Cardiovascular responses were not measured in Study 1 but we presume they would mirror the cortisol reactivity responses such that the short/short individuals would show the greatest cardiovascular reactivity as well. In contrast, the opposite genotype, the long/long genotype, was associated with greater cardiovascular reactivity in an initial study using the same emotional recall paradigm described above (Williams et al., 2008) as well as in a replication (Brummett et al., 2011). Thus, in the TSST studies, the Cys23 allele and the 5-HTTLPR short allele are associated with greater reactivity, while in the emotion recall paradigm the opposite alleles, the Ser23 allele and the 5-HTTLPR long allele, are associated with greater reactivity. Because both findings have been replicated, the consistency at the genetic level points to psychological differences between the two stressor paradigms as a more likely explanation for the differential relation of each allele to stress reactivity.

7.2. Psychological mechanisms

Although both the emotion recall (Brummett et al., 2014a, 2012) and our TSST studies used cortisol as a dependent measure, the tasks are psychologically very different. The two prior studies associating the Ser23 allele with greater cortisol reactivity asked participants to recall and relive emotional experiences (Brummett et al., 2014a, 2012). Specifically, participants spent 5 min reading emotionally neutral material in the presence of the experimenter before 5 min recalling an incident that made them extremely angry, followed by another 5 min of emotionally neutral public reading as before, and finally a 5 min recall of an experience that made them extremely sad. Five min of rest interspersed each of these manipulations. In contrast, in the present studies and in the companion paper (Avery and Vrshek-Schallhorn, 2016), participants were subject to social evaluation threat and performance pressure.

One psychological difference between the two paradigms that may help explain the differential allelic associations with cortisol reactivity is the degree of task controllability (Dickerson and Kemeny, 2004). In the emotion recall paradigm, the participant, by definition, has control over the experience recalled and its intensity. In contrast, both the speech and mental arithmetic components of the TSST are designed to induce a lack of perceived control. In the speech, the persistent negative reaction of the evaluative audience induces a sense the participant cannot control the outcome. Similarly, the forced fast pace and corrective interruptions from the evaluators during the mental arithmetic generate a sense of uncontrollability.

In animal models, the degree of control over a stressor has a robust effect on serotonin release (Maier and Watkins, 2005). For example, when a rat has behavioral control over termination of the stressor (i.e. shock) there is only a transient rise of extracellular serotonin in the vicinity of serotoninergic cell bodies (Dorsal Raphe nucleus; Amat et al., 2005) or terminal fields (i.e. amygdala; Amat et al., 1998). However, when a yoked rat receives the same shocks without behavioral control over the stressor, a persistent elevation in serotonin levels is observed. This activation of serotonin neurons is necessary and sufficient for the subsequent behavioral effects of uncontrollable stress, including reduced social exploration or deficits in escape behavior (Christianson and Greenwood, 2014). These behavioral effects of learned helplessness can be prevented by antagonists of the 5-HT_{2C} receptor (Christianson et al., 2010; Strong et al., 2009) or by physical exercise, which downregulates the 5-HT_{2C} receptor (Greenwood et al., 2012).

These differences in serotonin system signaling as a function of the controllability of the stressor in rodents may indicate that the TSST and emotion recall paradigms differentially activate the serotonin system. It would be expected that the greater uncontrollability of the TSST would lead to greater serotonin signaling than the more controllable emotion recall task. Differences in the magnitude of serotonin signaling between the two paradigms could change the direction of relation between a 5-HTTLPR or Cys23Ser allele and the phenotype. For example, signaling via the 5-HT_{2C} receptor appears to have an inverted U-shaped response. Low doses of a 5-HT_{2C} agonist have been shown to enhance the release of a neurotransmitter (dopamine) by pharmacological challenge (cocaine administration) while higher doses have been shown to have the opposite cellular effect, reducing the release of dopamine (Navailles et al., 2008). Accordingly, at the presumed low levels of 5-HT_{2C} stimulation elicited by the more controllable emotional recall paradigm employed by Brummett et al. (2014a, 2012), the Ser23 allele would be more responsive to stimulation, and thus lead to greater cortisol release. However, the greater 5-HT_{2C} stimulation elicited by the TSST due to its uncontrollability may be at the high end of the inverted U-shaped response curve for the Ser23 allele and lead to reduced cortisol response. According to this interpretation, the Cys23 allele would be closer to its peak response at the higher level of stimulation. How this model of controllability would affect serotoninergic regulation of the cortisol response to non-psychosocial stressors (e.g. physical pain; Mickey et al., 2012) is unclear.

In addition to differences in controllability between the two paradigms, there are differences in the affective states induced by the two paradigms. By design, the emotion recall paradigm induces sadness and anger, while the TSST elicits negative affect more broadly and particularly negative self-conscious emotions (e.g. shame; Dickerson, 2008). These potential differences in affect could indicate different patterns of neural activation associated with each of the tasks. These different networks could be regulated differently by the Cys23Ser polymorphism or 5-HTTLPR. For example, the same dose of 5-HT_{2C} agonist administered into the ventral tegmental area can have the opposite effect on dopamine neurons from that administered into the nucleus accumbens (Navailles et al., 2008). Such opposite effects of the 5-HT_{2C} receptor in different

brain regions could explain the effects seen here if the emotion recall task activates a circuit affected in one direction by a particular Cys23Ser or 5-HTTLPR allele and the TSST activates a circuit affected in the opposite direction by the same allele. Such effects have been seen in animal models. For example, in rodents, an antagonist of the 5-HT_{2C} receptor reduces impulsive choice in a delay discounting task, but increases impulsivity in the five-choice serial reaction time task, a measure of impulsive action (Higgins et al., 2013). Thus, blocking the 5-HT_{2C} receptor can have opposite behavioral effects depending on the paradigm used. By extension, genetic differences in the human 5-HT_{2C} receptor or serotonin transporter might lead to different responses to other seemingly similar tasks that involve different psychological processes and circuits, such as the emotional recall task and the TSST. Thus, this alternative mechanism focuses less on the magnitude of differences in serotoninergic activation between the two paradigms and more on the pattern of neural activation and its potential differential regulation by serotonin. Naturally, these hypotheses await empirical verification but they do suggest the importance of focusing on well-specified psychological processes in genetic association studies.

Social psychologists have long studied how situational factors alter or even reverse behavioral effects (Ross and Nisbett, 2011). However, there has been little exploration of the neurochemical mechanisms responsible for these effects. Situationally dependent associations between a genotype and phenotype such as reported here with the TSST and that reported previously with the emotion recall paradigm suggest that exploration of the relations between situational factors and neurochemical signaling is likely to be a valuable future avenue of research for both pharmacological and genetic association studies. Precisely delineating the psychological and neurochemical mechanisms for such discrepancies will be necessary for understanding the relevance of these gene variants for clinical outcomes.

7.3. Translational implications

The relation between reactivity to laboratory stressors and mental and physical health outcomes is not well understood. A large body of work has examined the reactivity hypothesis (Krantz and Manuck, 1984; Obrist, 1976) and found that heightened reactivity to laboratory stressors predicted adverse health outcomes, particularly cardiovascular ones (e.g., Hamer et al., 2012).Yet there is emerging evidence that *blunted* reactivity to laboratory stressors may also predict adverse health outcomes (Carroll et al., 2009; de Rooij, 2013; Phillips et al., 2013). For example, blunted cortisol reactivity to the TSST in soldiers prior to deployment predicted a greater increase in PTSD symptoms following deployment (Steudte-Schmiedgen et al., 2015). Similarly, early childhood abuse and adversity, a major risk factor for adulthood mental and physical health problems, has been linked in multiple studies to blunted cortisol reactivity to laboratory stressors (review: Lovallo, 2013). The most salubrious physiological stress reactivity profile may be a moderate one, neither unresponsive nor hyper-responsive to the stressor (Allen, 2013).

In terms of the Cys23Ser polymorphism, if the results presented here are viewed from the perspective of the reactivity hypothesis, the higher cortisol reactivity in individuals with the Cys23 genotype would predict more negative health outcomes. Alternatively, according to the blunted reactivity perspective, the low levels of cortisol reactivity in the Ser23 carriers would be predictive of poorer health outcomes. This would be consistent with epidemiological evidence that the Ser23 allele is a risk factor for myocardial infarction (Brummett et al., 2013) and depression when exposed to life stress (Brummett et al., 2014b).

The blunted cortisol reactivity in Ser23 carriers does not appear to extend to their subjective reaction to this stressor. We found a non-significant (marginal) increase in negative affect and anxiety in Ser23 carriers in Study 1 that was not replicated in Study 2. Avery and Vreshek-Schallhorn (2015) found a trend for greater negative self-conscious emotions in Ser23 carriers, but no association with negative affect. The lack of a relation between cortisol responses and subjective responses in these set of studies is not surprising in light of an early *meta*-analysis (Dickerson and Kemeny, 2004) and a more recent review of TSST studies (Campbell and Ehlert, 2012) that found no association between the measures. Brummett and colleagues (2012) found that the Ser23 males had greater depressed mood after the emotion recall task, but did not report general negative affect findings in their replication (Brummett et al., 2014a). Thus, there appears to be more consistency across the TSST and emotion recall paradigms for the association between the Cys23Ser polymorphism and affective responses than the association with cortisol responses. However these various results concerning self-reported affect should be interpreted cautiously as they are statistically weak, variable across studies, and based on different measures.

7.4. Limitations

Several limitations of these studies are noteworthy. In Study 1, psychiatric status was checked in a very simple way, through one item in the initial telephone screening survey querying diagnosed anxiety or depressive disorder within the last 6 months. So it is possible that some active psychiatric disorders were missed. The similarity of the cortisol findings across the two studies, and to those of Avery and Vrshek-Schallhorn (2016) wherein screening was done more thoroughly, suggests that the minimal level of exclusion did not impact the findings significantly. However additional research is needed to examine whether psychiatric diagnoses may interact with these polymorphisms to influence HPA axis responses to social evaluative threat and other social stressors.

Another limitation of research on the Cys23Ser polymorphism is the low frequency of the Ser23 allele. Although each study reported here, and in the companion paper (Avery and Vrshek-Schallhorn, 2016), had a higher frequency of Ser23 than the combination of the two prior experimental studies assessing the association of the Cys23Ser polymorphism with cortisol reactivity (Brummett et al., 2014a, 2012), the low number of Ser23 allele cases makes it difficult to reach a conclusive determination of the degree to which the relations reported here are due to either the Ser23 homozygotes, hemizygotes, or heterozygotes.

8. Conclusion

The consistency of the results across the two sites in this paper as well as the additional site in the companion paper (total $N = 426$) provides strong support for an association of the Cys23Ser polymorphism with cortisol reactivity in the TSST. Furthermore, replication of the 5-HTTLPR's association with cortisol reactivity provides additional evidence that this marker may influence the HPA axis. Taken together, these findings underscore the importance of the serotonin system in regulating cortisol reactivity to stress.

Contributors

Study 1 was designed and conducted by K.W.B., N.M., and J.Q. Study 2 was designed and conducted by S.T. B.M.W. developed the hypothesis and oversaw the genotyping procedures. Analyses were performed by K.W.B. All authors participated in the writing of the manuscript, which was initially drafted by B.M.W. and K.W.B.

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2016.04.022.

References

Allen, M.T., 2013. Integrative commentary: implications of blunted reactivity. Int. J. Psychophysiol. Off. J. Int. Organ. Psychophysiol. 90, 95–98, http://dx.doi.org/10.1016/j.ijpsycho.2013.07.012.

Amat, J., Matus-Amat, P., Watkins, L.R., Maier, S.F., 1998. Escapable and inescapable stress differentially and selectively alter extracellular levels of 5-HT in the ventral hippocampus and dorsal periaqueductal gray of the rat. Brain Res. 797, 12–22, http://dx.doi.org/10.1016/S0006-8993(98)00368-0.

Amat, J., Baratta, M.V., Paul, E., Bland, S.T., Watkins, L.R., Maier, S.F., 2005. Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. Nat. Neurosci. 8, 365–371, http://dx.doi.org/10.1038/nn1399.

Anchordoquy, H.C., McGeary, C., Liu, L., Krauter, K.S., Smolen, A., 2003. Genotyping of three candidate genes after whole-genome preamplification of DNA collected from buccal cells. Behav. Genet. 33, 73–78.

Avery, B.M., Vrshek-Schallhorn, S., 2016. Nonsynonymous HTC polymorphism predicts cortisol response to psychosocial stress I: effects in males and females. Psychoneuroendocrinology, http://dx.doi.org/10.1016/j.psyneuen.2015.12.023 (in press).

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinform. Oxf. Engl. 21, 263–265, http://dx.doi.org/10.1093/bioinformatics/bth457.

Brummett, B.H., Siegler, I.C., Ashley-Koch, A., Williams, R.B., 2011. Effects of 5HTTLPR on cardiovascular response to an emotional stressor. Psychosom. Med. 73, 318–322, http://dx.doi.org/10.1097/PSY.0b013e3182118c16.

Brummett, B.H., Kuhn, C.M., Boyle, S.H., Babyak, M.A., Siegler, I.C., Williams, R.B., 2012. Cortisol responses to emotional stress in men: association with a functional polymorphism in the 5HTR2C gene. Biol. Psychol. 89, 94–98, http://dx.doi.org/10.1016/j.biopsycho.2011.09.013.

Brummett, B.H., Babyak, M.A., Jiang, R., Shah, S.H., Becker, R.C., Haynes, C., Chryst-Ladd, M., Craig, D.M., Hauser, E.R., Siegler, I.C., Kuhn, C.M., Singh, A., Williams, R.B., 2013. A functional polymorphism in the 5HTR2C gene associated with stress responses also predicts incident cardiovascular events. PLoS One 8, e82781, http://dx.doi.org/10.1371/journal.pone.0082781.

Brummett, B.H., Babyak, M.A., Kuhn, C.M., Siegler, I.C., Williams, R.B., 2014a. A functional polymorphism in the HTR2C gene associated with stress responses: a validation study. Biol. Psychol. 103, 317–321, http://dx.doi.org/10.1016/j.biopsycho.2014.10.006.

Brummett, B.H., Babyak, M.A., Williams, R.B., Harris, K.M., Jiang, R., Kraus, W.E., Singh, A., Costa, P.T., Georgiades, A., Siegler, I.C., 2014b. A putatively functional polymorphism in the HTR2C gene is associated with depressive symptoms in white females reporting significant life stress. PLoS One 9, e114451, http://dx.doi.org/10.1371/journal.pone.0114451.

Carroll, D., Lovallo, W.R., Phillips, A.C., 2009. Are large physiological reactions to acute psychological stress always bad for health? Soc. Pers. Psychol. Compass 3, 725–743.

Chaouloff, F., Berton, O., Mormède, P., 1999. Serotonin and stress. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 21, 28S–32S, http://dx.doi.org/10.1016/S0893-133X(99)00008-1.

Christianson, J.P., Greenwood, B.N., 2014. Stress-protective neural circuits: not all roads lead through the prefrontal cortex. Stress 17, 1–12, http://dx.doi.org/10.3109/10253890.2013.794450.

Christianson, J.P., Ragole, T., Amat, J., Greenwood, B.N., Strong, P.V., Paul, E.D., Fleshner, M., Watkins, L.R., Maier, S.F., 2010. 5-Hydroxytryptamine 2C receptors in the basolateral amygdala are involved in the expression of anxiety after uncontrollable traumatic stress. Biol. Psychiatry 67, 339–345, http://dx.doi.org/10.1016/j.biopsych.2009.09.011.

de Rooij, S.R., 2013. Blunted cardiovascular and cortisol reactivity to acute psychological stress: a summary of results from the Dutch Famine Birth Cohort Study. Int. J. Psychophysiol. Off. J. Int. Organ. Psychophysiol. 90, 21–27, http://dx.doi.org/10.1016/j.ijpsycho.2012.09.011.

Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol. Bull. 130, 355–391, http://dx.doi.org/10.1037/0033-2909.130.3.355.

Dickerson, S.S., 2008. Emotional and physiological responses to social-evaluative threat. Soc. Personal. Psychol. Compass 2, 1362–1378.

Fentress, H.M., Grinde, E., Mazurkiewicz, J.E., Backstrom, J.R., Herrick-Davis, K., Sanders-Bush, E., 2005. Pharmacological properties of the Cys23Ser single nucleotide polymorphism in human 5-HT2C receptor isoforms. Pharmacogenom. J. 5, 244–254, http://dx.doi.org/10.1038/sj.tpj.6500315.

Gelernter, J., Kranzler, H., Cubells, J.F., 1997. Serotonin transporter protein (SLC6A4) allele and haplotype frequencies and linkage disequilibria in African- and European-American and Japanese populations and in alcohol-dependent subjects. Hum. Genet. 101, 243–246.

Greenwood, B.N., Strong, P.V., Loughridge, A.B., Day, H.E.W., Clark, P.J., Mika, A., Hellwinkel, J.E., Spence, K.G., Fleshner, M., 2012. 5-HT2C receptors in the basolateral amygdala and dorsal striatum are a novel target for the anxiolytic and antidepressant effects of exercise. PLoS One 7, e46118, http://dx.doi.org/10.1371/journal.pone.0046118.

Gruenewald, T.L., Kemeny, M.E., Aziz, N., Fahey, J.L., 2004. Acute threat to the social self: shame, social self-esteem, and cortisol activity. Psychosom. Med. 66, 915–924, http://dx.doi.org/10.1097/01.psy.0000143639.61693.ef.

Hamer, M., Endrighi, R., Venuraju, S.M., Lahiri, A., Steptoe, A., 2012. Cortisol responses to mental stress and the progression of coronary artery calcification in healthy men and women. PLoS One 7, e31356, http://dx.doi.org/10.1371/journal.pone.0031356.

Heils, A., Teufel, A., Petri, S., Stöber, G., Riederer, P., Bengel, D., Lesch, K.P., 1996. Allelic variation of human serotonin transporter gene expression. J. Neurochem. 66, 2621–2624.

Heisler, L.K., Pronchuk, N., Nonogaki, K., Zhou, L., Raber, J., Tung, L., Yeo, G.S.H., O', S., Rahilly, Colmers, W.F., Elmquist, J.K., Tecott, L.H., 2007. Serotonin activates the hypothalamic-pituitary-adrenal axis via serotonin 2C receptor stimulation. J. Neurosci. Off. J. Soc. Neurosci. 27, 6956–6964, http://dx.doi.org/10.1523/JNEUROSCI.2584-06.2007.

Hernando-Herraez, I., Prado-Martinez, J., Garg, P., Fernandez-Callejo, M., Heyn, H., Hvilsom, C., Navarro, A., Esteller, M., Sharp, A.J., Marques-Bonet, T., 2013. Dynamics of DNA methylation in recent human and great ape evolution. PLoS Genet. 9, e1003763, http://dx.doi.org/10.1371/journal.pgen.1003763.

Higgins, G.A., Sellers, E.M., Fletcher, P.J., 2013. From obesity to substance abuse: therapeutic opportunities for 5-HT2C receptor agonists. Trends Pharmacol. Sci. 34, 560–570, http://dx.doi.org/10.1016/j.tips.2013.08.001.

Jahnsen, J.A., Uhlén, S., 2012. The N-terminal region of the human 5-HT_2C receptor has as a cleavable signal peptide. Eur. J. Pharmacol. 684, 44–50, http://dx.doi.org/10.1016/j.ejphar.2012.03.043.

Kahn, R.S., Kalus, O., Wetzler, S., Cahn, W., Asnis, G.M., van Praag, H.M., 1990. Effects of serotonin antagonists on m-chlorophenylpiperazine-mediated responses in normal subjects. Psychiatry Res. 33, 189–198.

Kaiser, R., Tremblay, P.B., Roots, I., Brockmöller, J., 2002. Validity of PCR with emphasis on variable number of tandem repeat analysis. Clin. Biochem. 35, 49–56.

Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The trier social stress test—a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28, 76–81.

Krantz, D.S., Manuck, S.B., 1984. Acute psychophysiologic reactivity and risk of cardiovascular disease: a review and methodologic critique. Psychol. Bull. 96, 435–464.

Lappalainen, J., Zhang, L., Dean, M., Oz, M., Ozaki, N., Yu, D.H., Virkkunen, M., Weight, F., Linnoila, M., Goldman, D., 1995. Identification, expression, and pharmacology of a Cys23-Ser23 substitution in the human 5-HT2c receptor gene (HTR2C). Genomics 27, 274–279, http://dx.doi.org/10.1006/geno.1995.1042.

Lehman, B.J., Cane, A.C., Tallon, S.J., Smith, S.F., 2015. Physiological and emotional responses to subjective social evaluative threat in daily life. Anxiety Stress Coping 28, 321–339, http://dx.doi.org/10.1080/10615806.2014.968563.

Liposits, Z., Phelix, C., Paull, W.K., 1987. Synaptic interaction of serotonergic axons and corticotropin releasing factor (CRF) synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. A light and electron microscopic immunocytochemical study. Histochemistry 86, 541–549.

Lovallo, W.R., 2013. Early life adversity reduces stress reactivity and enhances impulsive behavior: implications for health behaviors. Int. J. Psychophysiol. Off. J. Int. Organ. Psychophysiol. 90, 8–16, http://dx.doi.org/10.1016/j.ijpsycho.2012.10.006.

Maier, S.F., Watkins, L.R., 2005. Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. Neurosci. Biobehav. Rev. 29, 829–841, http://dx.doi.org/10.1016/j.neubiorev.2005.03.021.

McNair, D.M., Lorr, M., Droppleman, L.F., 1971. Profile of Mood States. Educational and Industrial Testing Service, San Diego, CA.

Mickey, B.J., Sanford, B.J., Love, T.M., Shen, P.-H., Hodgkinson, C.A., Stohler, C.S., Goldman, D., Zubieta, J.-K., 2012. Striatal dopamine release and genetic variation of the serotonin 2C receptor in humans. J. Neurosci. Off. J. Soc. Neurosci. 32, 9344–9350, http://dx.doi.org/10.1523/JNEUROSCI.1260-12.2012.

Miller, R., Wankerl, M., Stalder, T., Kirschbaum, C., Alexander, N., 2013. The serotonin transporter gene-linked polymorphic region (5-HTTLPR) and cortisol stress reactivity: a meta-analysis. Mol. Psychiatry 18, 1018–1024, http://dx.doi.org/10.1038/mp.2012.124.

Mueller, A., Armbruster, D., Moser, D.A., Canli, T., Lesch, K.-P., Brocke, B., Kirschbaum, C., 2011. Interaction of serotonin transporter gene-linked polymorphic region and stressful life events predicts cortisol stress response. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 36, 1332–1339, http://dx.doi.org/10.1038/npp.2011.11.

Navailles, S., Moison, D., Cunningham, K.A., Spampinato, U., 2008. Differential regulation of the mesoaccumbens dopamine circuit by serotonin2C receptors in the ventral tegmental area and the nucleus accumbens: an in vivo microdialysis study with cocaine. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 33, 237–246, http://dx.doi.org/10.1038/sj.npp.1301414.

Obrist, P.A., 1976. Presidential Address: 1975. The cardiovascular-behavioral interaction—as it appears today. Psychophysiology 13, 95–107.

Okada, M., Northup, J.K., Ozaki, N., Russell, J.T., Linnoila, M., Goldman, D., 2004. Modification of human 5-HT(2C) receptor function by Cys23Ser, an abundant, naturally occurring amino-acid substitution. Mol. Psychiatry 9, 55–64, http://dx.doi.org/10.1038/sj.mp.4001357.

Peciña, M., Azhar, H., Love, T.M., Lu, T., Fredrickson, B.L., Stohler, C.S., Zubieta, J.-K., 2013. Personality trait predictors of placebo analgesia and neurobiological correlates. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 38, 639–646, http://dx.doi.org/10.1038/npp.2012.227.

Pereg, D., Gow, R., Mosseri, M., Lishner, M., Rieder, M., Van Uum, S., Koren, G., 2011. Hair cortisol and the risk for acute myocardial infarction in adult men. Stress 14, 73–81, http://dx.doi.org/10.3109/10253890.2010.511352.

Phillips, A.C., Ginty, A.T., Hughes, B.M., 2013. The other side of the coin: blunted cardiovascular and cortisol reactivity are associated with negative health outcomes. Int. J. Psychophysiol. Off. J. Int. Organ. Psychophysiol. 90, 1–7, http://dx.doi.org/10.1016/j.ijpsycho.2013.02.002.

Ross, L., Nisbett, R.E., 2011. The Person and the Situation: Perspectives of Social Psychology. Pinter & Martin Publishers.

Seibyl, J.P., Krystal, J.H., Price, L.H., Woods, S.W., D', C., Amico, Heninger, G.R., Charney, D.S., 1991. Effects of ritanserin on the behavioral neuroendocrine, and cardiovascular responses to meta-chlorophenylpiperazine in healthy human subjects. Psychiatry Res. 38, 227–236.

Slatkin, M., 2008. Linkage disequilibrium—understanding the evolutionary past and mapping the medical future. Nat. Rev. Genet. 9, 477–485, http://dx.doi.org/10.1038/nrg2361.

Smith, T.W., Birmingham, W., Uchino, B.N., 2012. Evaluative threat and ambulatory blood pressure: cardiovascular effects of social stress in daily experience. Health Psychol. Off. J. Div. Health Psychol. Am. Psychol. Assoc., http://dx.doi.org/10.1037/a0026947.

Stetler, C., Miller, G.E., 2011. Depression and hypothalamic-pituitary-adrenal activation: a quantitative summary of four decades of research. Psychosom. Med. 73, 114–126, http://dx.doi.org/10.1097/PSY.0b013e31820ad12b.

Steudte-Schmiedgen, S., Stalder, T., Schönfeld, S., Wittchen, H.-U., Trautmann, S., Alexander, N., Miller, R., Kirschbaum, C., 2015. Hair cortisol concentrations and cortisol stress reactivity predict PTSD symptom increase after trauma exposure during military deployment. Psychoneuroendocrinology 59, 123–133, http://dx.doi.org/10.1016/j.psyneuen.2015.05.007.

Strong, P.V., Greenwood, B.N., Fleshner, M., 2009. The effects of the selective 5-HT(2C) receptor antagonist SB 242084 on learned helplessness in male Fischer 344 rats. Psychopharmacology 203, 665–675, http://dx.doi.org/10.1007/s00213-008-1413-3.

Vijayendran, M., Cutrona, C., Beach, S.R.H., Brody, G.H., Russell, D., Philibert, R.A., 2012. The relationship of the serotonin transporter (SLC6A4) extra long variant to gene expression in an African American sample. Am. J. Med. Genet. Part B Neuropsychiatr. Genet. Off. Publ. Int. Soc. Psychiatr. Genet. 159B, 611–612, http://dx.doi.org/10.1002/ajmg.b.32054.

Walstab, J., Steinhagen, F., Brüss, M., Göthert, M., Bönisch, H., 2011. Differences between human wild-type and C23S variant 5-HT2C receptors in inverse agonist-induced resensitization. Pharmacol. Rep. PR 63, 45–53.

Watson, D., Clark, L.A., Tellegen, A., 1988. Development and validation of brief measures of positive and negative affect: the PANAS scales. J. Pers. Soc. Psychol. 54, 1063–1070.

Way, B.M., Taylor, S.E., 2010. The serotonin transporter promoter polymorphism is associated with cortisol response to psychosocial stress. Biol. Psychiatry 67, 487–492, http://dx.doi.org/10.1016/j.biopsych.2009.10.021.

Way, B.M., Taylor, S.E., 2011. A polymorphism in the serotonin transporter gene moderates cardiovascular reactivity to psychosocial stress. Psychosom. Med. 73, 310–317, http://dx.doi.org/10.1097/PSY.0b013e31821195ed.

Way, B.M., Lacan, G., Fairbanks, L.A., Melega, W.P., 2007. Architectonic distribution of the serotonin transporter within the orbitofrontal cortex of the vervet monkey. Neuroscience 148, 937–948, http://dx.doi.org/10.1016/j.neuroscience.2007.06.038.

Wigginton, J.E., Cutler, D.J., Abecasis, G.R., 2005. A note on exact tests of Hardy-Weinberg equilibrium. Am. J. Hum. Genet. 76, 887–893.

Williams, R.B., Marchuk, D.A., Siegler, I.C., Barefoot, J.C., Helms, M.J., Brummett, B.H., Surwit, R.S., Lane, J.D., Kuhn, C.M., Gadde, K.M., Ashley-Koch, A., Svenson, I.K., Schanberg, S.M., 2008. Childhood socioeconomic status and serotonin transporter gene polymorphism enhance cardiovascular reactivity to mental stress. Psychosom. Med. 70, 32–39, http://dx.doi.org/10.1097/PSY.0b013e31815f66c3.

Yonan, A.L., Palmer, A.A., Gilliam, T.C., 2006. Hardy-Weinberg disequilibrium identified genotyping error of the serotonin transporter (SLC6A4) promoter polymorphism. Psychiatr. Genet. 16, 31–34.