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A Functional Polymorphism in the *HTR2C* Gene Associated with Stress Responses: A validation study

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Abstract

Previously we have shown that a functional nonsynonymous single nucleotide polymorphism (SNP), rs6318 on the *HTR2C* gene located on the X-chromosome, is associated with hypothalamic-pituitary-adrenal axis response to a laboratory stress recall task. The present paper reports a validation of the cortisol response to stress in a second, independent sample. The study population consisted of 60 adult participants (73.3% males). Consistent with our prior findings, compared to Cys23 G allele carriers, persons homozygous for the Ser23 C allele had a significantly greater average cortisol response ($p = 0.007$) and area under the curve ($p = .021$) over the course of an emotional stress recall protocol. Also parallel to our prior report, the change in cortisol from baseline to the average during the stress protocol was roughly twice as large among Ser23 C homozygotes than among persons with Cys23 G. These findings validate our initial observation of association between rs6318 and cortisol response to an acute stressor, and extend the results to include females.

Keywords

rs6318; *HTR2C*; stress

Introduction

HTR2C is a widely investigated serotonin receptor (Martina, Michel, Lanfumey, & Mongeau, 2014). The role it plays with respect to numerous critical neural circuits makes it a strong candidate for genetic association studies of behavior. For example, *HTR2C* has been associated with psychiatric disorders, feeding behavior, and antipsychotic-induced side effects (Drago & Serretti, 2009). An *HTR2C* variant that has received considerable attention

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is the rs6318 single nucleotide polymorphism (SNP), an x-linked nonsynonymous coding SNP. This SNP is known to lead to a substitution of serine for cysteine at codon 23 (Cys23Ser), and is constitutively more active than the Cys23 G allele (Okada et al., 2004). The rs6318 variant is among those demonstrating the most consistent results in association studies (Drago & Serretti, 2009). The frequency of the Ser23 C allele is approximately 13% in unrelated Caucasians (Lappalainen et al., 1995).

The *HTR2C* receptor plays a key role in the mediation of stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation by the release of serotonin in the central nervous system (CNS) (Dinan, 1996; Heisler et al., 2007; Lowry, 2002). We recently reported that in a sample of males *HTR2C* rs6318 was associated with serum cortisol responses to a series of emotional stress tasks in the laboratory (Brummett et al., 2012). Specifically, Ser23 C allele hemizygotes (men carry only one copy of the allele) had two-fold larger increases in plasma cortisol during recall of situations that made them angry or sad compared to those carrying the Cys23 G allele. This finding corroborated existing evidence that the *HTR2C* receptor plays a key role in the activation of the HPA axis by acute stress, and is consistent with prior research (Okada, et al., 2004) showing the Ser23 C allele is constitutively more active than the Cys23 G allele. This prior study, however, consisted of males only. In related recent study of 6,126 men and women referred for diagnostic cardiac catheterization, we also showed that over a 7-year follow-up period, males hemizygous for Ser23 C and females homozygous for Ser23 C allele had an increase in cardiovascular disease event (death and/or MI) risk (hazard ratio = 1.38) compared to those with other rs6318 genotypes (Brummett et al., 2013). One possible mechanism that might explain the increased prognostic risk associated with Ser23C might be the tendency to respond to psychological stress during daily life with heightened HPA-axis activity, which, over time is thought to promote atherogenesis (Rozanski, Blumenthal, Davidson, Saab, & Kubzansky, 2005). The association between cortisol responses to laboratory stress and cortisol levels throughout the day has not been studied extensively. Recent findings indicate that cortisol responses assessed via laboratory stress are positively associated with cortisol area under the curve (AUC) levels during the day (Kidd, Carvalho, & Steptoe, 2014). Moreover, this association is apparently independent of sex, age, socioeconomic status, smoking, body mass index, and time of laboratory testing. Thus, acute laboratory stress testing has ecological validity.

Reviewing candidate gene by environment interaction studies in the field of psychiatric research, Duncan and Keller (Duncan & Keller, 2011) concluded that in order to advance the field, studies conducted with the intent of closely replicating prior work should be considered greater scientific merit than isolated novel findings. In this context the current study provides a direct attempt to validate and build upon previous results. The sample used in the present report were participants in a different part of the same larger study from which the sample for the earlier Brummett et al. paper (Brummett, et al., 2012) were derived. The present sample is an entirely different group of participants from those used the prior paper. The stress protocol was carried out in the same laboratory by the same workers. Our aim was to validate our prior finding that, compared to Cys23 G, Ser23 C was associated with a heightened cortisol response to a laboratory stressor. The present study also extends the prior work by including women.

Methods

Participants

Participants were recruited to take part in a study designed to examine the moderating effects of genetic, behavioral, and environmental mechanisms on health disparities. The study was conducted at Duke University Medical Center, and all subjects gave informed consent prior to their participation in the study using a form approved by the Duke University Medical Center Institutional Review Board. The current study sample consisted of participants who were part of a tryptophan depletion/enhancement study. Data for the present study was taken from the “sham” control condition of the depletion arm, in which participants drank a “milkshake” fortified with a full complement of amino acids. Sixty two participants with complete genetic and cortisol data were available for analysis. Because the *HTR2C* gene is located on the X chromosome we examined only men and homozygous women.

Procedure

See below and also our prior papers (Brummett, et al., 2012; Williams et al., 2001, 2003) for a full description of study procedures. Briefly, upon evening admission to the General Clinical Research Unit at Duke University Medical Center, sociodemographic data were gathered and blood was drawn for assessment of biological parameters. Test day one: the focus of the present study--consisted of a sham tryptophan depletion (in contrast to a sham tryptophan *enhancement* that was the focus of our prior study (Brummett, et al., 2012) followed by an emotional stress reactivity protocol (see, (Williams, et al., 2001). On the day of the sham depletion, beginning at 6:30 a.m. participants were not allowed food or liquids, but were required to consume a milkshake that consisted of all essential amino acids. The protocol timing is as follows: From 7:00 a.m. till noon reclining rest period → At noon basal baseline cortisol blood sample drawn followed by a 45 minute break and participants were informed that the stress protocol would begin shortly → At 12:45 the 45-minute stress protocol and begins and proceeds as follows:

5-minute anticipatory “protocol” baseline/ 5-minute public speaking task / 5-minute rest / 5-minute anger induction / 5-minute rest / 2nd 5-minute public reading task / 5-minute rest / Sadness induction / Recovery. Blood samples were drawn for cortisol assay after the basal and protocol baseline and each task and rest period thereafter.

During the 5-minute baseline participants were instructed to remain seated and quiet, and that the stress portion of the study would begin in 5 minutes. Next participants were asked to read aloud for 5 minutes text from emotionally neutral material while in the presence of the experimenter (who was the same individual for all participants). The reading period was followed by a 5 minutes of rest during which the participant was instructed to remain quiet. During the anger induction, lasting 5 minutes, participants are asked to recall an incident that made them extremely angry. Participants are then asked to recount, relive, and describe in detail that incident to the experimenter. The anger induction is then followed by another 5-minute rest period, 5-minute public reading task, and 5-minute rest. This is followed by the sadness induction which is identical to the anger induction with the exception that the

participant is asked to recall an incident that made them extremely sad. The stress protocol is then concluded with a 5-minute recovery period during which the participants are informed that the stress protocol has been completed and they are asked to remain seated and quiet.

Cortisol

Blood samples were spun for 15 minutes in a refrigerated centrifuge and plasma was transferred into polypropylene tubes containing .05 ml glutathione and then frozen at -70°C . Blood samples were processed at the Clinical Research Unit at DUMC under the supervision of Dr. Cynthia Kuhn. Cortisol was measured by specific radioimmunoassay (RIA) using Coat-A-Count kit from Diagnostic Products Inc. Inter-/Intra-assay coefficients of variation were less than 10% and 5%, respectively.

Genotyping

Genomic DNA was extracted for genotyping by standard procedure (Puregene D-50K isolation kit, Gentra, Minneapolis, MN) from fresh or frozen samples of peripheral blood collected from the participants. The SNP, rs6318, was identified from NCBI's Single Nucleotide Polymorphism database (dbSNP) (<http://www.ncbi.nlm.nih.gov/SNP>). Genotyping was accomplished using the ABI 7900 Taqman system (Applied Biosystems, Carlsbad, California). Each marker, including rs6318, was required to achieve 95% efficiency (at least 95% of the genotypes could be scored with certainty). A series of blinded QC samples were also included for that required a 100% match for the marker to be considered for statistical analysis. Finally, rs6318 met the assumptions for Hardy-Weinberg Equilibrium ($p=0.29$). Heterozygous women were excluded from the analysis, yielding two genotype groups, male hemizygotes and female homozygotes with Ser23 C or Cys23 G.

Statistical Analyses

Genotype group differences on baseline characteristics were tested using t-tests. In parallel with our previous approach, a repeated measures mixed model (PROC MIXED, SAS, version 9.2, Cary NC) was used to examine the association of rs6318 genotype with cortisol changes over the course of the stress protocol. Specifically, rs6318 allelic variation (C vs G) was examined as a predictor of the course of cortisol response across the alternating stress and rest time periods, adjusted for basal cortisol levels (obtained at 12:00 noon following the lengthy rest period). Cortisol was transformed using natural logarithm in all analyses to better meet the distributional assumptions of model residuals. The mixed model also included age, sex, and race as adjustment variables. Because resting cortisol is used as a covariate, the main effect for allele carries information about the difference between the allele types *averaged across the cortisol measurements subsequent to, and adjusted for the baseline*. A time by genotype interaction term examined whether the genotype groups differed in the pattern of postprotocol baseline cortisol response. The interaction term was removed from the model if not substantial or statistically significant. We also compared the rs6318 genotype groups on the cortisol area under the curve (AUCi) (Fekedulegn et al., 2007), calculated using the trapezoid method, beginning with the protocol baseline measure up to and including the final resting measure at the end of the stress protocol. We used the general linear model to compare the rs6318 allelic variation (Ser23C vs Cys23G) on AUCi.

The model again included age, sex, and race, and basal cortisol levels as adjustment covariates.

Results

The sample consisted of 60 adults (8 white women, 19 white men, 8 black women, 25 black men). Fifteen participants (25.0%) were Ser23 C allele carriers. The mean age was 33.7 years (SD=8.7) among the Ser23 C group and 34.7 years (SD=7.7) among the Cys23 G group ($p=.64$). The median basal cortisol was 63.7 pg/ml (interquartile range [IQR]= 49.8) in the Ser23 C group compared to 60.7 pg/ml (IQR=35.0) in the Cys23 G group, $p = .71$). The raw cortisol levels across the stress protocol for each genotype are displayed in Figure 1. The mixed model revealed a main effect for genotype, $p = .007$. Calculating the geometric mean cortisol level from the log-transformed values, the mean cortisol across the stress protocol, adjusted for age, race, sex, and basal cortisol, was 120.3 pg/ml (log-transformed value = 4.79, standard error=0.09) in the Ser23 C group, compared to 90.0 pg/ml (log-transformed value = 4.50, standard error=.05) in the Cys23 G group. The change in cortisol from the basal baseline to the average level during the stress protocol was a little more than twice as large in the Ser23 C group, 55.7 pg/ml, compared to 25.3 pg/ml in the Cys23 G group. The time by genotype interaction was not statistically significant, $p = 0.27$.

Comparing the genotypes on cortisol AUCi, the adjusted mean AUCi was 933 (95% CI= 754, 1112) in the Ser23C group compared to 706 (95% CI=616, 796) in the Cys23G group. The difference between the two groups was 227 (95% CI = 35, 419), which was statistically significant ($p = .021$). The sample standard deviation for AUCi was 319. Thus the standardized difference between genotypes was $227/319 = 0.71$.

One anonymous reviewer requested that we also report our results separately for each sex, acknowledging that power would likely be insufficient among women. Among the 16 females available for analysis, only one had the risk genotype (homozygous for Ser23C). In the mixed model, that CC woman had an average (log transformed) cortisol of 5.0 (95% CI = 4.4, 5.7) pg/ml during the postbaseline tasks, while the average of the 15 G carriers was 4.6 (95% CI = 4.4, 4.8) pg/ml, $p = .17$. For men, the 14 C hemizygotes had an average post-baseline cortisol level of 4.7 (95% CI = 4.5, 4.9), compared to 4.4 (95% CI = 4.3, 4.5) pg/ml for the 30 G hemizygous males ($p = .023$). With respect to AUC, the sole CC woman had an AUC of 1082 (95% CI = 385, 1780), while the G carriers' AUC was 781 (95% CI = 607, 957), $p = .38$. Male Cs had an AUC of 863 (95% CI = 698, 1029), while the AUC for Male Gs was 654 (95% CI = 551, 758).

Discussion

Consistent with our prior findings, we have observed in an independent sample that the cortisol response to laboratory stress tasks was substantially larger among men and women with the Ser23 C allele compared to those carrying the Cys23 G allele. This result was consistent for cortisol response quantified as the mean level or as AUCi. Thus, we have provided further support for the proposition that the 5HTR2C receptor plays a role in the activation of the HPA axis by acute stress. Psychological stress in daily life is associated

with a higher cortisol awakening response and higher mean day and evening cortisol levels (e.g., Kumari et al., 2010), and as noted earlier, cortisol response to laboratory stressors has been shown to correspond to cortisol response in daily life (Kidd, et al., 2014). Thus the identification of genes that might control cortisol reactivity to laboratory stressors also may help explain individual variation in cortisol response during daily life.

Investigation of the neurobiological mechanisms by which psychosocial risk factors enhance the activity of the HPA axis indicates that stress-induced release of serotonin in the CNS acts at multiple brain sites to contribute to stress-induced HPA axis activation. These sites include limbic forebrain structures--the prefrontal cortex, amygdala, and hypothalamus. In the hypothalamus serotonin stimulates HTR2C receptors on cells in the paraventricular nucleus (PVN), co-expressing corticotrophin releasing hormone (CRH), which is released when the HTR2C receptors are stimulated (Dinan, 1996; Heisler, et al., 2007; Lowry, 2002). The present findings, along with studies linking the Ser23 C allele with affective disorders, also suggest that the rs6318 effects on HT2C receptor-mediated effects on emotion and the HPA axis may moderate, at least in part, the linkages between depression and dysregulated HPA axis function. In addition, the present findings also may point to a mechanism underlying the association we previously reported between Ser23 C and incidence of death or MI in a large CHD patient sample (Brummett, et al., 2013).

Our findings also may offer some insight into why the Ser23 C variant has also been found to be associated with affective disorders such as depression (Drago & Serretti, 2009; Lerer et al., 2001). Depression has long been linked to dysregulated HPA axis function. For example, depressed men exhibit higher levels of salivary cortisol across the day compared to healthy men (Hinkelmann et al., 2012). Moreover, cortisol is also thought to be a distinguishing factor between two major subtypes of depression--melancholic and atypical. Melancholic depression is more likely to be associated with raised levels of cortisol, whereas atypical depression is more often associated with decreased cortisol levels (Gold & Chrousos, 2002, 2013). The present results that suggest rs6318 variation is associated with increased cortisol responding. We might therefore speculate that the Ser23 C allele would likely be more closely related to depression with melancholic features than to atypical depression. Indeed, examining associations of several polymorphisms with subphenotypes of major depression, Hahn et al. (Hahn et al., 2008) showed that rs6318 Ser23 C carriers were significantly more likely to manifest somatic symptoms of anxiety—an indicator of melancholic depression—as compared to Cys23 G carriers. This finding, however, was no longer statistically significant when adjusted for multiple tests. Nevertheless, taken together, the above suggests that a potentially fruitful area for future investigation would be to include examination of different subtypes of depression with respect to rs6318 and cortisol response.

In surveying other studies in order to compare the effect size of rs6318 on cortisol response from the present study with the extant literature, we found considerable variation among stress induction methods, cortisol assay and quantification, analytic approaches, and availability of information required for effect size calculation. We were, nevertheless, able to identify several studies of cortisol, stress, and relevant genes that provided sufficient information for the calculation of effect size. Two of these studies used laboratory stress procedures somewhat similar to ours. In the first, Armbruster et al. (Taylor, Larson, &

Lauby, 2014) studied several polymorphisms on the *HT2A* receptor gene and found a standardized effect size (Cohen's *d*) for genotype of about 0.46 ($\eta^2=.05$) for the baseline to peak salivary cortisol difference, a value similar to our effect size of 0.43 for baseline to protocol average cortisol. Second, Kumsta et al. (Kumsta, Chen, Pape, & Heinrichs, 2013) also used a laboratory stress protocol to study salivary cortisol response with respect to polymorphisms on *NPSRI*, a functional gene that control neuropeptide S. The typical observed effect sizes explained between 2% and 3% of the variance in the average cortisol response from baseline, which can be roughly translated to standardized differences of 0.30 to .035; smaller, but again, not too different from our own estimates. Using a much more demanding stress challenge, Taylor et al. (Mahon, Zandi, Potash, Nestadt, & Wand, 2013) studied salivary cortisol response differences between variants of the serotonin transporter gene during a weeklong military survival exercise. Their findings comparing the variants can be translated as a standardized difference in cortisol response of about 0.41, again quite similar to ours. Colzato et al. (Colzato, Van der Does, Kouwenhoven, Elzinga, & Hommel, 2011) used a cold pressor task to study the effect of the Val66Met variant on the *BDNF* gene on cortisol response. Interestingly, the genotype group differed significantly on cortisol levels during the pre-stress anticipatory period, but not after the stressor had been applied. The standardized effect size for the anticipatory measurement was roughly 0.26, with the minor allele Met carriers showing greater cortisol increases compared to Val homozygotes. Finally, Goodyer et al. (Goodyer, Croudace, Dudbridge, Ban, & Herbert, 2010) examined both the *BDNF* Val66Met and *5HTTLPR* variants in adolescent boys and girls with respect to morning cortisol levels. No genetic difference was observed for *BDNF*, but the s' allele on *5HTTLPR* was associated with higher levels of morning cortisol, with a standardized effect size of roughly 0.32. While this brief survey of effect sizes is not intended to be exhaustive, it does suggest that despite considerable methodological variation across studies, our effect size is comparable to those seen in other studies of similar variables.

An important limitation of the current study is the small sample size and consequent limited number of participants carrying the rs6318 C allele. However, the present study results are quite similar to those in our prior study with an independent sample, providing encouraging evidence that the phenomenon is robust. An additional limitation is that ancestry markers were unavailable in this sample and we were thus not able to account for population stratification. Consequently, we cannot rule out confounding due to stratification. We note again, however, that concordance between this and the prior study, despite at least minor differences in ethnic makeup strengthens the case that the result is not an artifact. Finally, the modest sample size precluded reliable statistical tests of moderation by gender, race, or other possible strata. We note, however, that descriptive analysis of these data showed that the pattern of higher cortisol stress response in Ser23C individuals was consistent within each subgroup of men, women, Blacks, and Whites. It is also possible that the sham tryptophan depletion drink itself might alter cortisol response, obscuring or altering the effects of the stress tasks. However, the results of studies that have included a sham tryptophan depletion and assessed cortisol response indicate that sham depletion has no significant effect on cortisol response levels (Goddard et al., 1995; Price, Malison, McDougale, Pelton, & Heninger, 1998). These potential concerns notwithstanding, having demonstrated a similar pattern of cortisol response in two independent samples, a next step

in studying rs6318 might include the use of gene-expression analysis and possibly pharmacological probes to further evaluate the genetic contribution of the cortisol response. For example, HTR2C sensitivity might be manipulated with an agonist or antagonist, and the differential response to stress recorded as a function of genotype and the presence or absence of the probe. In the longer term, the current findings may inform the development of targeted preventive interventions and treatments of affective disorders, such as depression, and more speculatively, coronary heart disease.

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Highlights

- Cortisol response to stress in the laboratory was associated with an *HTR2C* variant
- Ser23C carriers had 2-fold greater cortisol response than Cys23G carriers
- Results validate prior findings that were in an independent sample of men
- The present results extend the previous work by including females

Figure.

Unadjusted cortisol levels for rs6318 genotype groups in response to a laboratory stress protocol. Solid horizontal lines in boxes are medians; notches indicate approximate 95% confidence intervals of median; hinges represent 25th and 75th percentile; upper and lower whiskers are $1.5 \times$ interquartile range; dots represent outliers. Results of a mixed model, adjusting for age, sex, race, and basal (resting) cortisol levels showed main effect for genotype, $p = 0.014$.