Nonsynonymous HTR2C Polymorphism Predicts Cortisol Response to Psychosocial Stress I: Effects in Males and Females

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Abstract:

Background Genetic influences on stress reactivity may provide insight into depression risk mechanisms. The C-allele of rs6318, a putatively functional polymorphism located within the HTR2C gene, has been reported to predict greater cortisol and negative affective reactivity to lab-induced stress. However, the potential moderating effect of sex has not been examined despite X-linkage of HTR2C. We hypothesized that sex moderates the effect of rs6318 on cortisol and affective reactivity to lab-induced stress, with males showing stronger effects.

Methods Non-depressed young adults (N = 112; 39 female) screened via clinical interview provided a DNA sample and completed either a negative evaluative Trier Social Stress Test, or a non-evaluative control protocol. Salivary cortisol and self-reported affect were assessed at four timepoints.

Results Contrary to hypotheses, C-carriers showed blunted rather than exaggerated cortisol responses to lab-induced stress in multilevel models ($b = 0.467$, $p < 0.001$), which persisted when covarying subclinical depressive symptoms. This effect was not moderated by sex ($b = 0.174$, $p = 0.421$), and remained significant when examining females ($b = 0.362$, $p = 0.013$) and males ($b = 0.537$, $p < 0.001$) separately. C-carriers also exhibited marginally greater reactivity in negative self-focused affect in response to stress than non-carriers when covarying subclinical depressive symptoms ($b = -0.360$, $p = 0.067$), and exhibited higher levels of subclinical depressive symptoms than non-carriers ($F = 6.463$, $p = 0.012$).

Conclusions Results support a role for the rs6318 C-allele in dysregulated stress responding, and suggest that the C-allele may contribute to risk for depression.

Keywords: rs6318 | cortisol | serotonin | gene-environement interaction | lab-induced stress | major depressive disorder

Article:

1. Introduction

Research investigating neuroendocrine reactivity to laboratory-induced stress has demonstrated that risk factors for depression predict dysregulated cortisol responding to psychosocial stress (e.g., Oswald et al., 2006, Wirtz et al., 2007) and that genetic factors account for a moderate amount of variance in cortisol reactivity (Federenko et al., 2004, Steptoe et al., 2009). Furthermore, lab-induced psychosocial stress has been used to demonstrate gene-environment (G × E) interactions (e.g., Miller et al., 2013) that meta-analytic evidence suggests also occur naturalistically (Karg et al., 2011). Thus, lab-induced psychosocial stress studies may provide novel insights into genetic risk for depression.

One particular serotonin receptor, 5-HT2C, is implicated in stress responding and risk for depression: 5-HT2C knockout mice show reduced anxiety-related behaviors (Heisler et al., 2007b) and a novel 5-HT2C antagonist is efficacious for treating depression (Loo et al., 2002). Recently, Brummett et al. (2012) reported that a putatively functional single-nucleotide polymorphism (SNP), rs6318, in the gene encoding the 5-HT2C receptor, HTR2C, predicted greater cortisol and negative affect in response to a lab-induced stress protocol in a sample of 41 males. This SNP comprises a G to C switch at basepair 68, conferring a serine for cysteine substitution at codon 23 of the HTR2C gene, which is located on the X chromosome (Lappalainen et al., 1995). The C-allele confers greater 5-HT2C activity (Okada et al., 2004), consistent with the notion that higher 5-HT2C activity level is associated with more pronounced stress responding. Brummett et al. (2012) found that C-allele genotype males exhibited larger increases in cortisol blood concentration and negative affect than G-allele genotype males. Additionally this finding was replicated in a sample (N = 60) that included C/C (n = 1) and G/G (n = 15) females, although females were not tested separately (Brummett et al., 2014a). In a larger sample, the same group also found that the C-allele moderates the relationship between self-reported life stress and depressive symptoms in C/C females, but not in C males (Brummett et al., 2014b), consistent with prior work that implicated the C-allele in risk for both depression and bipolar disorder (Lerer et al., 2001).

Despite the small sample sizes previously assessed, the possibility that rs6318 contributes to stress responses is intriguing. Putatively a functional SNP, the C-allele is associated with greater 5-HT2C receptor activity in COS-7 cells transfected with human DNA (Okada et al., 2004). Furthermore, there is accumulating evidence that serotonergic systems, including the 5-HT2C receptor, biologically interact with the hypothalamic–pituitary–adrenal (HPA) axis both in animal models and in humans (for a review, see Lanfumey et al., 2008). For instance, administration of a 5-HT2C agonist, m-cholorophenylpiperazine, resulted in increased activity of neurons containing corticotropin-releasing hormone (CRH) in the paraventricular nucleus of mice (Heisler et al., 2007a,) and elevated ACTH and cortisol levels in humans (for a review, see Kahn and Wetzler, 1991). Additionally, hypothalamic brain slices of 5-HT2C knockout mice failed to demonstrate expected increases in CRH secretion seen in control mice following stress, indicating that 5-HT2C receptor activation may stimulate increased HPA activity (Heisler et al., 2007b). Finally, administration of a selective 5-HT2C antagonist, FR260010, (Harada et al., 2006) attenuated a conditioned behavioral fear response in rats that were previously exposed to a robust stressor (Harada et al., 2008). Taken together, the 5-HT2C receptor appears to play a key role in regulating cortisol and behavioral responses to stress. Furthermore, given reported effects of rs6318 on

negative affect under stress and evidence for its functionality, the C-allele may contribute to risk for emotional disorders such as depression, either alone or in interaction with life stress (Brummett et al., 2014b).

However, despite the observed effect of the rs6318 SNP on stress responses in males, neither an independent replication in males nor evidence for an effect in females alone have been reported. Furthermore, the relationship of rs6318 to cortisol stress-responses has not been examined in a sample that contains C/G females, who are more common than C/C females. There are several reasons to believe that effects of rs6318 may be attenuated in females, the majority of whom have either the G/G or C/G genotype. In females, one X-chromosome per cell is sequestered at random as an inactive Barr body (Barr and Bertram, 1949, Lyon, 1961). Because rs6318 is X-linked, the effects of rs6318 may be significantly attenuated in females as compared to males, representing a gene by environment by sex interaction. Consistent with the possibility of a sex difference in this genetic effect, twin research suggests that a related construct, liability to depression (Kendler and Prescott, 1999), does not perfectly overlap in males and females ($r = 0.57$). Alternatively, expression of the C-allele by 50% of cells in C/G females, who make up a majority of C-carriers, may be sufficient to produce a similar magnitude of response as seen in males. Although Brummett et al. (2014a) replicated their finding that the C-allele confers greater cortisol reactivity to stress in a sample that included sixteen females, only one female in this sample had the C/C genotype, and none had the C/G genotype (Brummett et al., 2014a). Thus, the role of biological sex in moderating the effects of rs6318 on the stress response has not been sufficiently examined.

1.1. The Present study

Here, we examined the role of rs6318 and sex on cortisol and affect in response to a negative evaluative Trier Social Stress Test (TSST; Kirschbaum et al., 1993) or a control protocol in currently non-depressed young adults. In addition to assessing broad negative affect, we also assessed negative self-focused affect (i.e., shame and guilt) due to evidence that it is particularly perturbed under negative evaluative stress (Dickerson et al., 2004). First, we hypothesized that rs6318 C-carriers would show significantly greater cortisol and affective responses to negative evaluative lab-induced stress than their non-C-carrier counterparts, and that the effect of this SNP would be significantly less pronounced in the control condition, representing a gene-environment interaction. Second, we hypothesized that biological sex would further moderate this interaction, with females showing a significantly attenuated effect of genotype on cortisol and affective responses, yielding a three-way interaction. Third, due to evidence that dysregulation of the serotonergic and HPA systems are associated with risk for depression (Karg et al., 2011, Miller et al., 2013), we conducted an exploratory analysis examining whether the C-allele would be associated with higher rates of current subclinical depressive symptoms.

2. Method

2.1. Participants

Participants were recruited from the Introductory Psychology pool at a midsize Midwestern private university. Prior to enrollment, participants were screened for eligibility during a mass testing session. Eligible participants were at least 18 years of age, and denied having chronic health

conditions or using nicotine, hormonal birth control, prescription psychotropic medications, or corticosteroid medications. Additionally, due to meta-analytic findings that current clinically significant depression is associated with blunted cortisol responses to lab-induced stress (Burke et al., 2005), we only included individuals who were currently non-depressed at the time of the TSST, ascertained via both questionnaire screening and clinical interviews as described below. Finally, due to cognitive performance measures not reported here, additional participation criteria included speaking English as a first language, having normal or corrected-to-normal vision and hearing, and denying colorblindness, diagnosed learning disability, and any history of head trauma.

Informed consent for the larger study was provided by 127 participants. Of these, 114 also consented to provide a saliva sample for DNA analyses. Participants were assigned to experimental conditions pseudo-randomly (60 controls, 54 Stress condition); testing sessions were scheduled in advance as either Stress or Control sessions, and participants were blind to the pre-assigned condition when signing up for timeslots. One participant in the Stress condition withdrew from the study after receiving instructions for the negative evaluative TSST, and one control participant's session could not be completed due to interruption by a fire evacuation, leaving 112 participants (39 female) who completed the study and provided signed permission for their data to be used following debriefing (for sample characteristics, see Table 1). One male participant was excluded from cortisol analyses due to consistent outlying cortisol concentration values, but was included in the analyses of affect. Additionally, one male participant did not provide affect ratings due to technical difficulties, but was included in the analyses of cortisol. Participants ranged in age from 18 to 22 years (M = 18.70, SD = 0.815); 83 participants (74.1%) were Caucasian and 29 (25.9%) participants were a minority race or ethnicity. All study sessions began at either 1:00 p.m. or 3:30 p.m. to reduce the likelihood that naturally high morning cortisol levels might obscure cortisol responses to the TSST.

Table 1. Sample characteristics.

Sex	Condition	rs6318 Genotype		
		C	G	
Male	Stress	6	35	
	Control	3	29	
		C/C	C/G	G/G
Female	Stress	1	6	11
	Control	0	3	18

2.2. Materials

2.2.1. Questionnaires

Prior to enrollment, participants were screened for depression using the symptom questions of the Diagnostic Inventory for Depression (DID; Zimmerman et al., 2004), a nineteen-item measure that assesses the severity of the nine symptoms of a major depressive episode (MDE) described in the DSM-5 on a scale from 0 to 4. We excluded two items assessing suicidal ideation because it was not feasible to ensure the safety of individuals who might report thoughts of self-harm, leaving seventeen items assessing eight symptoms of an MDE. Because we lacked the suicidal ideation symptom, we screened out individuals who endorsed four (instead of five) or more MDE symptoms at a level of two or higher, consistent with scoring recommendations. We also excluded

individuals who answered insufficient items to rule out an MDE classification. In addition to using this measure as a screening tool, in several analyses, we used DID scores among participants as a dimensional measure of subclinical depression symptom severity, ranging from no symptoms to a level approaching a diagnosis of depression.

At four points during the study (baseline immediately prior to the TSST, and approximately 20, 45 and 65 minutes following the baseline assessment), participants completed the Positive and Negative Affect Schedule (PANAS; Watson et al., 1988) and the Guilt subscale from the PANAS-Expanded Form (PANAS-X; Watson and Clark, 1999) to capture negative self-focused affect. The PANAS includes two 10-item mood scales assessing positive and negative affect; the self-focused affect subscale consists of six items measuring negative affect associated with shame and guilt. Participants rated all items on a five-point Likert scale ranging from one (very slightly or not at all) to five (extremely). Because two items from the Guilt subscale measuring negative self-focused affect are also part of the negative affect scale of the PANAS, we excluded these items from the calculation of negative affect scores to prevent spurious overlap between negative affect and self-focused affect subscales.

2.2.2. Salivary cortisol

To assess cortisol levels, participants provided saliva samples via passive drool into sterile cryogenic vials at four points following completion of the PANAS scales. All samples were stored in a −20 °C freezer within 20 minutes of the end of each session where they were maintained until the end of data collection. Samples were then packed in dry ice and shipped to Trier, Germany, where they were assayed using time-resolved fluorescent-detection immunoassay (DELFIA; Dressendörfer et al., 1992). Intra-assay variation ranged from 4.0% to 6.7%, and inter-assay variation ranged from 7.1% to 9.0%. Cortisol data were logarithmically transformed prior to conducting analyses but are depicted as raw values in graphs presented here.

2.2.3. Clinical interviews

Following enrollment in the study, participants completed the MDE section of the Structured Clinical Interview for DSM-IV, non-patient edition (SCID-I/NP; First et al., 2001) to assess past depressive episodes and ensure that participants had not developed a depressive episode between screening and study enrollment. The interview was conducted by trained undergraduate research assistants who demonstrated proficiency conducting the MDE section of the interview during mock interviews with a licensed clinical psychologist (SVS) and matched a set of internally developed gold standard ratings. All diagnoses were reviewed during group supervision meetings with the principal investigator. Interviewers blind to initial diagnoses provided diagnoses of audiorecorded interviews to assess inter-rater reliability for past MDEs, $k = 0.88$ (adjusted for equiprobability); there were no diagnosed cases of current MDEs.

2.2.4. Genotyping

Participants provided an additional saliva sample for DNA analyses. DNA was extracted using the Promega Maxwell 16 tissue DNA purification kit (Promega Corporation, Madison, WI) and genotyped for rs6318 using a Taqman SNP Genotyping Assay kit on Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA). To examine the effects of the C-allele,

genotypes were coded as C-carriers (in males C, in females either C/G or C/C) or not (in males G, in females G/G) using values of 1 and 0, respectively. Analyses were repeated excluding one C/C female participant to ensure that results were not due to this individual.

2.3. Procedure

All procedures were approved by the University's Institutional Review Board. Participants were told that the study examined responses to challenging situations, and that the investigators would not reveal everything about the challenge protocol upfront, but that they would be debriefed at the end of the study. After the informed consent process and DNA collection, participants completed the MDE section of the SCID-I/NP, followed by several computerized questionnaires (data not reported here). Participants then completed the negative evaluative TSST or control protocol, followed by three questionnaire items intended to assess the effectiveness of the experimental manipulation. These items inquired about the extent to which participants felt evaluated during the task and, if so, the extent to which that evaluation was positive or negative. Next, participants completed two cognitive tasks lasting approximately 25 minutes (data not reported here) and were then debriefed and given a few minutes to rest. Participants completed the PANAS assessment and provided saliva samples at four points: at baseline prior to the TSST or control protocol, immediately afterward, following two cognitive tasks, and following debriefing plus several minutes of rest. Thus, affect was assessed at approximately 0, +20, +45, and +65 minutes relative to the beginning of the TSST or control protocol. PANAS negative affect following debriefing was compared with baseline levels in order to ensure that participants were not upset by the procedures. All participants provided signed permission to use the data following debriefing.

2.3.1. Trier Social Stress Test

Participants completed either a negative evaluative TSST or a no-audience control protocol adapted from Way and Taylor (2010). Consistent with the traditional TSST (Kirschbaum et al., 1993), in both conditions, participants were given instructions for the task, followed by a five-minute preparation period for the speech, a five-minute period during which participants spoke on an assigned topic, and a five-minute arithmetic task during which participants were instructed to serially subtract 13 from 2017 out loud. In the arithmetic task, participants in both conditions were informed of their errors and instructed to start over when they made mistakes. All participants were told that they would be videotaped during the task and each completed the task while looking into a video camera that was ostensibly recording; however, participants were not actually recorded.

The control protocol differed from the negative evaluative TSST in several ways. Participants in the Control condition were told they would not be evaluated, were given a non-evaluative speech topic (healthy lifestyle tips others could follow), and had no audience. The experimenter sat out of the participant's line of sight following the instructions, only speaking politely when necessary to request that the participant continue trying to speak for the entire five minutes of the speech portion or point out mistakes during the mental arithmetic portion. By contrast, participants in the Stress condition were given an evaluative speech topic (why they should be chosen by their peers for a student leadership position) and spoke to a panel of two confederates (one male, one female) posing as judges. Confederates followed a behavioral script to provide negative non-verbal feedback throughout the speech and arithmetic tasks. Participants were told that these confederates were trained in the analysis of non-verbal aspects of public

speaking, that they would be evaluating their speech for content and delivery style, and that the video recording of the speech would later be subjected to voice-frequency analysis and an analysis of non-verbal behaviors by public speaking experts.

2.4. Statistical approach

To examine how genotype and sex related to changes in cortisol and affect over the course of the experiment, we conducted growth curve analyses using multilevel regression models (MRM; cf. hierarchical linear modeling) in SAS 9.3 PROC MIXED with maximum likelihood estimation (Raudenbush and Bryk, 2002, Singer and Willett, 2003). In keeping with the expected increase and subsequent decline in cortisol and negative affect following the TSST, and in keeping with prior work (e.g., Zoccola et al., 2008), our hypotheses and interpretations focused on the curvilinear (i.e., quadratic) component of the changes in cortisol and affect over time. We refer to this quadratic effect as reactivity throughout the results. All models also accounted for linear change in dependent variables. Separate analyses examined salivary cortisol, negative affect, and the self-focused affect subscales as dependent variables.

To test the hypotheses that C-carriers would exhibit greater physiological stress and negative affective reactivity in the context of stress, we examined three-way interactions of Genotype × Stress (experimental condition) × Quadratic Time. Additionally, to examine whether the effect of the rs6318 SNP was moderated by sex, we examined a four-way interaction of Genotype × Stress × Quadratic Time × Sex. Meta-analytic findings indicate that individuals with current MDD exhibit blunted cortisol reactivity to lab-induced stress (Burke et al., 2005). Therefore, to ensure that results were not driven by differences in current subclinical depressive symptoms, we re-conducted the analyses using DID score as a covariate, including covarying all appropriate interaction terms to statistically remove its influence from the three-way interactions. Finally, we conducted a one-way ANOVA to examine whether there was an effect of rs6318 genotype on current subclinical depressive symptoms.

3. Results

3.1. Preliminary tests

Comparisons between pseudo-randomly assigned conditions indicated that the conditions were balanced. The Control condition did not differ from the Stress condition on minority status, sex, or history of a clinically significant major depressive episode (all $\chi 2(1) \leq 2.473$, ps ≥ 0.168). Additionally, conditions did not differ in body mass index, total DID score, baseline levels of cortisol, or baseline PANAS negative affect or self-focused affect (all Fs ≤ 1.221, all ps ≥ 0.272).

3.2. Manipulation checks

Participant responses to questionnaire items administered immediately after the TSST indicated that participants in the Stress condition felt more evaluated ($F(1,110) = 51.205$, $p < 0.001$), and felt that these evaluations were more negative ($F(1,104) = 87.419$, $p < 0.001$) and less positive ($F(1,104) = 27.265$, $p < 0.001$) than participants in the Control condition. In addition, a significant Stress × Quadratic Time interaction indicated that the Stress condition exhibited more pronounced cortisol reactivity compared to the Control condition ($b = -0.26$, $SE(b) = 0.041$, $t(213) = -6.30$, p

< 0.001) (see Table 2). Additionally, there was a significant Stress × Quadratic Time interaction on PANAS negative affect (b = −0.268, SE(b) = 0.0782, t(317) = −3.43, p < 0.001), where participants in the Stress condition exhibited greater reactivity in negative affect over time. This two-way interaction was not significant when examining the self-focused affect PANAS subscale (b = −0.050, SE(b) = .071, t(317) = −0.71, p = 0.477), suggesting that on average, there were no differences by condition in self-focused affect reactivity over time.

Table 2. Fixed Effects Results of Multilevel Regression Model predicting Cortisol Level.

Effect	b	SE (b)	df	t	p
Linear time	−0.199	0.054	107	−3.71	0.0003
Quadratic time	0.003	0.029	213	0.10	0.9187
Stress	0.374	0.096	213	3.9	0.0001
Stress × Linear Time	0.423	0.075	213	5.61	<0.0001
Stress × Quadratic Time	−0.260	0.041	213	−6.3	<0.0001
Genotype	−0.054	0.150	213	−0.36	0.7179
Genotype × Linear Time	−0.001	0.117	213	−0.01	0.993
Genotype × Quadratic Time	−0.135	0.064	213	−2.12	0.0351
Genotype × Stress	−0.469	0.250	213	−1.88	0.0619
Genotype × Stress × Linear Time	−0.208	0.196	213	−1.07	0.288
Genotype × Stress × Quadratic Time	0.467	0.107	213	4.39	<0.0001

3.3. Effects of rs6318

Contrary to hypotheses, a significant Genotype × Stress × Quadratic Time interaction indicated that rs6318 C-carriers relative to non-carriers exhibited blunted cortisol reactivity under negative evaluative stress compared to the Control condition (b = 0.467, SE(b) = 0.107, t(213) = 4.39, p < 0.001) (Fig. 1). This three-way interaction remained significant when controlling for subclinical depressive symptoms (DID symptom score) and its component interactions (b = 0.454, SE(b) = 0.118, t(208) = 3.87, p < 0.001). To decompose this significant three-way interaction, we examined the effect of Genotype × Quadratic Time separately in each condition. Within the Stress condition, participants who were C-carriers exhibited significantly blunted reactivity in cortisol over time (b = 0.332, SE(b) = 0.095, t(102) = 3.51, p < 0.001) compared to non-carriers. Conversely, within the Control condition, there was a significant Genotype × Quadratic Time interaction, where rs6318 C-carriers exhibited slightly but significantly greater reactivity in cortisol as a function of time than did non-carriers (b = −0.135, SE(b) = 0.056, t(111) = −2.39, p = 0.018) (Fig. 1).

3.4. Influence of sex on cortisol reactivity G × E

Also contrary to hypotheses, when sex was added to the model, the effect of rs6318 as a function of stress did not significantly vary by sex; that is, the four-way interaction of Sex × Genotype × Stress × Quadratic Time on cortisol was not significant (b = 0.174, SE(b) = 0.216, t(209) = 0.81, p = 0.421). Given this non-significant interaction effect, we examined whether the three-way Genotype × Stress × Quadratic Time interaction was detectable in each sex separately. There was a significant Genotype × Stress × Quadratic Time interaction in females (b = 0.362, SE(b) = 0.143, t(73) = 2.54, p = 0.013) (Fig. 2a) and also in males (b = 0.537, SE(b) = 0.157, t(136) = 3.42, p < 0.001) (Fig. 2b).

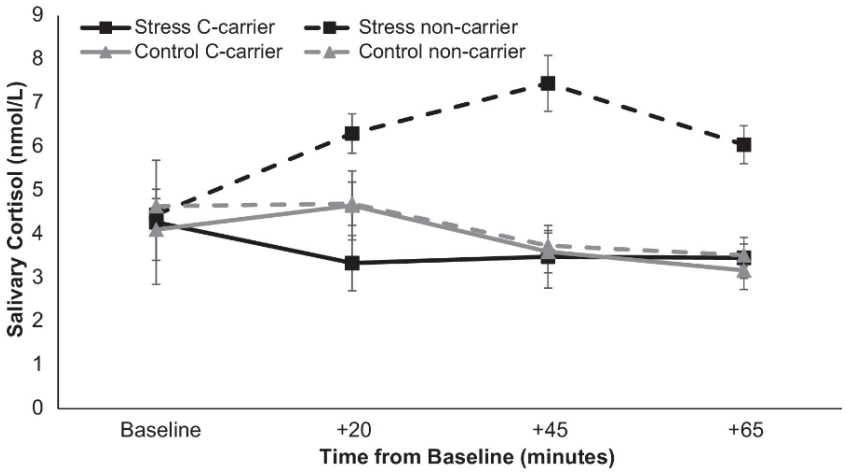
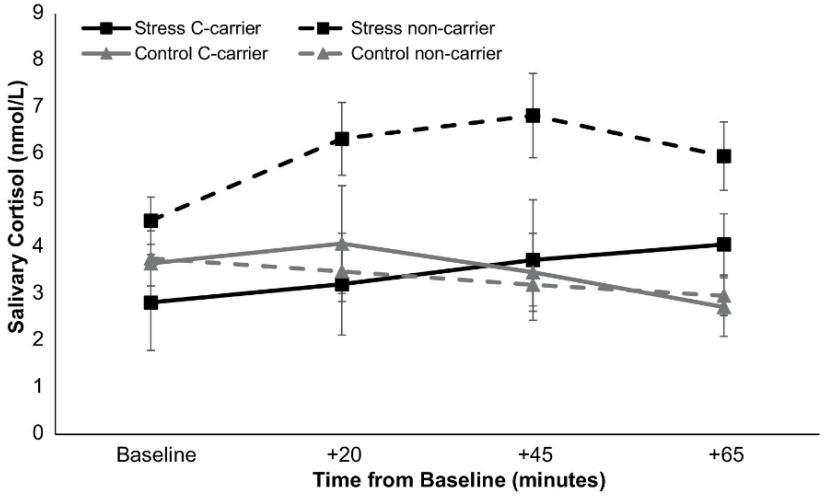


Fig. 1. Genotype × Stress × Time interaction: all participants.

a. Females



b. Males

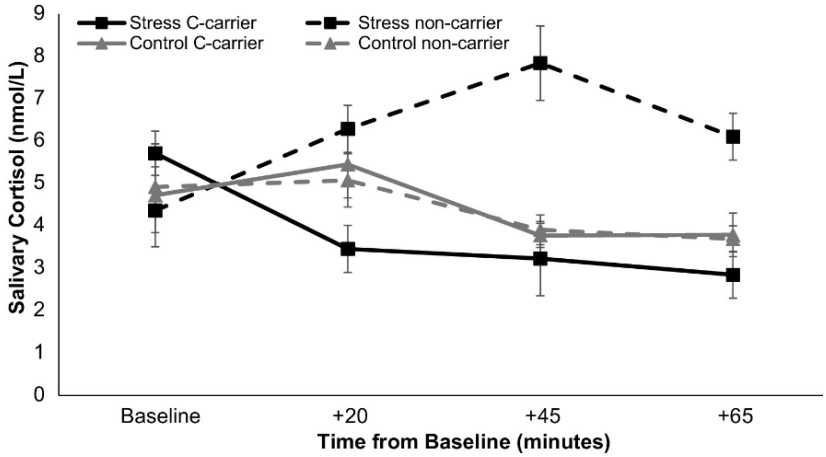


Fig. 2. Genotype × Stress × Time interaction separately by sex.

3.5. Affective reactivity

There was a marginal three-way Genotype × Stress × Quadratic Time interaction, where rs6318 C-carriers exhibited greater reactivity in self-focused affect under stress than under control conditions when covarying subclinical depressive symptoms ($b = -0.360$, $SE(b) = 0.198$, $t(317) = -1.82$, $p = 0.067$). However, this interaction did not approach significance without covarying subclinical depression ($b = -0.132$, $SE(b) = 0.090$, $t(322) = -1.46$, $p = 0.146$). We did not decompose this interaction because it did not reach traditionally accepted levels of significance. This three-way interaction was not significant for negative affect ($b = -0.178$, $SE(b) = 0.220$, $t(317) = -0.81$, $p = 0.420$). Given that neither of these effects reached conventional levels of significance, we did not examine the three way interaction of genotype, stress, and sex.1

3.6. Subclinical depressive symptoms

A one-way ANOVA revealed a significant main effect of genotype on subclinical depressive symptoms ($F(1,110) = 6.463$, $p = 0.012$). Specifically, C-carriers exhibited significantly higher DID scores, indicating higher levels of subclinical depressive symptoms ($M = 7.84$, $SD = 3.55$) than non-carriers ($M = 5.58$, $SD = 3.53$).

4. Discussion

Here we demonstrate that both non-depressed males and females carrying the C-allele of a putatively functional polymorphism (rs6318) in the serotonin 2c receptor gene, HTR2C, show blunted cortisol reactivity and heightened self-focused affect in response to a negative evaluative lab-based stressor versus a control protocol. We also show that this polymorphism is significantly associated with rates of subclinical depressive symptoms. Together with Way, Taylor, and Brown (under review), these papers represent the first set of independent conceptual replications of two previous reports that rs6318 C-carriers have dysregulated stress responses.

These results are particularly striking because the pattern of cortisol responses was opposite that previously reported in two samples (Brummett et al., 2014a, Brummett et al., 2012). Several differences in samples and procedures may have contributed to these divergent findings. First, although the procedures used in previous studies (Brummett et al., 2014a, Brummett et al., 2012) shared common features with the TSST (e.g., participants were asked to complete a 5-minute public speaking task), these procedures also included separate anger and sadness induction portions. Thus, the results of Brummett et al. (2012) may reflect individuals who were experiencing a more generalized negative affective state than participants in the present study, who engaged in a task aimed to solely induce stress resulting from social evaluative threat. Second, the previous studies examined community-based samples (Brummett et al., 2014a, Brummett et al., 2012), while the present study examined an undergraduate college population. Thus, the participants in previous studies (Brummett et al., 2014a, Brummett et al., 2012) were approximately 15–16 years older on average than the participants in the present study, and may have also differed on characteristics such as socioeconomic status and types of life stressors they had experienced. However, another recent study found no differences in the cortisol response to stress between a sample of students with a mean age of 21 years and a sample of university employees with a mean age of 38 years (Way et al., under review). Thus, it is unlikely that age differences between the present sample and the samples reported by Brummett et al., 2014a,

Brummett et al., 2012 account for the opposite pattern of results. Last, our protocol differed from previous studies in its methodology for the assessment of cortisol levels; previous studies measured cortisol in the bloodstream (Brummett et al., 2014a,2012,2), while the present study measured salivary cortisol. However, although each of these methods has relative advantages and disadvantages, (Kirschbaum and Hellhammer, 2000, Levine et al., 2007), both are widely accepted as valid methods of assessment, and there are no data to suggest that collection source might lead to results in opposite directions.

Although the direction of the relationship between genotype and cortisol reactivity was opposite our hypotheses, for several reasons we believe that the C-allele of rs6318 contributes to maladaptive stress responses as originally reported by Brummett et al. (2012). First, two other findings in this study support that C-carriers have more robust negative emotional reactivity than non-carriers: C-carriers display marginally greater increases in self-focused affect in response to lab stress and greater levels of subclinical depressive symptoms. We do not believe these findings are due to problems in genotyping in either the current or past studies: minor allele frequencies were similar across all studies, and genotypes for females in the present study do not deviate from Hardy–Weinberg Equilibrium. Second, past studies show that risk factors for depression can be associated with either exaggerated or blunted cortisol responses to lab stress. For example, neuroticism, low introversion, perfectionism (Wirtz et al., 2007), and stress-related rumination (Zoccola et al., 2010) predicted exaggerated cortisol responses. By contrast, in other work, high neuroticism and low extraversion were also associated with blunted cortisol responses (Oswald et al., 2006), as was depressive rumination (Zoccola et al., 2008). Therefore, although C-carriers showed significantly blunted cortisol responses to negative evaluative stress, these findings are still consistent with the hypothesis that the rs6318 C-allele is associated with dysregulation of HPA functioning.

We also predicted that the influence of the C-allele would be significantly attenuated in females because the 5-HT2C gene is X-linked, and in any given cell one X chromosome is sequestered at random. In contrast to our prediction, we did not find evidence that the effect of rs6318 was significantly weaker in females. Instead, we found that when examining females separately, there was a significant effect of rs6318 allele on cortisol reactivity to stress.

4.1. Association of rs6318 with subclinical depressive symptoms

Although we hypothesized that C-carriers would have greater evidence of dysfunctional affective functioning, and rs6318 has been shown to moderate the relationship between life stress and depression in C/C females (Brummett et al., 2014b), we were surprised to find in the exploratory analysis that C-carriers had significantly higher rates of current subclinical depressive symptoms. Historically, genetic main effects on depression in genome wide association studies have not reached significance (MDD Working Group of the GWAS Consortium, 2013) and studies examining the main effects of polymorphisms on depression have produced inconsistent results (for a review, see Lohoff, 2010). One possible conclusion based on these findings is that a majority of genetic variants in depression act in interaction with stress, rather than independently of it. For example, although two separate meta-analyses indicate that 5-HTTLPR is associated with dysregulated cortisol responses to stress (Miller et al., 2013) and shows a significant interaction with stress predicting depression (Karg et al., 2011), there are no recent meta-analyses supporting a genetic main effect on depression. We speculate that we found a significant main effect of genotype on subclinical depressive symptoms in part because we examined (dimensional)

subclinical symptoms as opposed to diagnoses, which ought to aid power while reflecting the same underlying construct as diagnosed depression (Kendler and Gardner, 1998). Additionally, the effect may have emerged in part because the sample was composed of undergraduates enrolled in a competitive, private university who may be prone to higher levels of perfectionism (Hibbard and Davies, 2011) and thus, higher rates of depressive conditions (Hewitt and Flett, 1990), also potentially boosting power. Such a main effect may not reliably be observed with clinically significant diagnoses of MDD, although we note that it has at times been observed (Lerer et al., 2001). In addition, there may be an underlying G × E effect with naturalistic stress (e.g., childhood adversity) that we did not account for, given that we did not assess naturalistic stress. Thus, although the rs6318 SNP appears promising, and evidence of an effect of the C-allele on subclinical depressive symptoms strengthens the interpretation that the C-allele is riskier than the G-allele, we suggest that future research on rs6318 should focus on gene-environment interactions rather than exclusively examining its main effects.

4.2. Directions for future research

Life stress and serotonergic system dysfunction have been implicated in many psychological disorders, especially the internalizing disorders, including Major Depressive Disorder (Ressler and Nemeroff, 2000), Generalized Anxiety Disorder (Ressler and Nemeroff, 2000), and Post-Traumatic Stress Disorder (Southwick et al., 1999). These findings strengthen evidence for the notion that HPA axis functioning is one mechanism through which serotonergic genetic variants confer greater risk for the onset of psychopathology in the context of life stress (Gotlib et al., 2008, Miller et al., 2013). Future research should further examine the effect of the rs6318 SNP in conjunction with life stress in precipitating these disorders. More specifically, research should examine how the rs6318 SNP might interact with life stress to predict dysfunctional profiles of HPA activity in response to stress observed in internalizing disorders.

Additionally, the inconsistency of the direction of our results with those of Brummett et al., 2014a, Brummett et al., 2014b, Brummett et al., 2012 highlights a prevalent theme in the literature on HPA dysregulation. Researchers have found that life stress, risk factors for psychopathology, and dysregulated HPA function is associated with both blunted (e.g., Oswald et al., 2006) and more robust (e.g., Wirtz et al., 2007) cortisol responses to lab-induced stress. Conceptualizing cortisol as a resource mobilizing hormone (Fries et al., 2009), one possible reason for these discrepancies may be that both more robust and blunted profiles of reactivity to stress are indicative of HPA dysfunction, but that blunted profiles of reactivity may be indicative of greater state levels of perceived helplessness. That is, a lack of physiological mobilization in response to stress may be the result of perceived helplessness or hopelessness in the face of a stressor, particularly negative evaluative threat, such as our manipulation employed. Future research should examine the basis of these discrepancies in the literature. Additionally, it is our hope that our findings, along with those of Brummett et al., 2014a, Brummett et al., 2014b, Brummett et al., 2012 and Way et al. (under review) will reinvigorate basic research on the biological connections between serotonergic and HPA systems, as both are strongly implicated in internalizing disorders.

4.3. Limitations

Although our study has several notable strengths, including examination of a biomarker, lab-standardized stress, exclusion of currently depressed individuals, inclusion of both males and

females, and the use of clinical diagnostic interviews for depression, it also has several limitations. First, the sample size is modest (N = 112), and includes a relatively small number of C-carriers (n = 19 in total). Thus, although we found significant effects of rs6318 on the cortisol response to stress in both sexes, our analyses examining interactions with sex may have been underpowered. Notably, the effect size estimate for the influence of the C-allele on the cortisol response to stress was somewhat lower in females than males, and the interaction of Sex × Genotype × Stress may be statistically significant in larger samples. However, the sample is larger than the two existing reports for the same SNP (N = 41 and N = 60), and is a similar size as other studies examining genetic effects on cortisol responses to lab-based stress (N = 67–518; Miller et al., 2013).

Second, sample limitations prevented us from examining potential differences in stress reactivity and depressive symptoms among females who carried one versus two rs6318 C alleles. Because only one female in the sample was homozygous for the C allele, results may not be generalizable to females with the CC genotype. Third, we did not collect a measure of naturalistic life stress for this sample, making it impossible to examine whether the main effect of rs6318 on current subclinical depressive symptoms could be accounted for by a gene-environment interaction effect instead of a main effect of this SNP. Future studies should pursue this approach.

4.4. Conclusions

Taken together, in a sample of non-depressed young adult males and females, we show that the rs6318 C-allele is associated with dysregulated neuroendocrine and affective stress responses, and elevated subclinical depressive symptoms. Thus, rs6318 is a promising SNP for investigation in gene-environment interactions for emotional disorders.

Conflict of interest

The authors report no conflicts of interest.

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Disclosure

S.V.S. designed the study and collected the data; B.M.A. and SVS analyzed the data; B.M.A. wrote the paper and S.V.S. edited it. All authors approved the final article.

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