

The Serotonin Transporter Promoter Polymorphism Is Associated with Cortisol Response to Psychosocial Stress

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Background: Across multiple mental health-related measures, a polymorphism (5-HTTLPR) within the promoter of the serotonin transporter gene has been associated with differential psychological sensitivity to stressful experiences. Yet, the specific mechanisms by which this polymorphism contributes to risk for psychological dysfunction is unclear. Therefore, we investigated cortisol reactivity to psychosocial stress as a potential intermediate phenotype that might predispose to such risk.

Methods: A psychologically healthy sample of 182 young adults were genotyped for the 5-HTTLPR. Each participant delivered a speech and performed mental arithmetic in one of three audience conditions: a critical evaluative audience, a supportive evaluative audience, or no audience. Salivary cortisol was sampled at baseline and at 20, 40, and 75 min after stressor onset.

Results: The two evaluative audience conditions elicited similar, significant increases in cortisol that were significantly greater than in the no audience control. Together, the evaluative audience conditions revealed a significant relationship between cortisol reactivity and the 5-HTTLPR, with the short/short genotype showing the greatest reactivity. Internal analyses revealed that the 5-HTTLPR was significantly associated with cortisol reactivity in the negative audience condition only, suggesting that short/short individuals might be especially vulnerable to social threat.

Conclusions: The short/short genotype of the 5-HTTLPR is associated with greater cortisol reactivity to social threat. When short/short individuals experience stressful life events, they might be at greater risk for the adverse psychological and physical health consequences associated with heightened cortisol exposure.

Key Words: Cortisol, mental health disorders, serotonin, stress

Although it is well-documented that exposure to stressful life experiences increases risk for adverse physical and mental health outcomes (1), this stress-related increase in risk is not uniform. Accumulating evidence indicates that genetic variation is a key contributor to individual variability in response to stressful life events (2). One marker of such variation is a polymorphism (5-HTTLPR) within the promoter of the serotonin transporter gene (*SLC6A4*). At this locus, there are two principal alleles, short and long, that differ in length by 43 base pairs (bp) (3,4).

A growing body of research suggests that the 5-HTTLPR moderates the effects of adverse life experiences on the probability and severity of a diverse array of mental health related conditions and constructs, including depression (5,6), suicide (7,8), anxiety (9,10), and substance abuse (11), to name a few. Across these diverse stress-related psychological disorders, most studies associate the 5-HTTLPR short allele and particularly the short/short genotype with greater psychological sensitivity to stress (12), although there is variability in the success of replicating such findings (13). One potential explanation for the variability in results is that the 5-HTTLPR is more closely associated with the regulation of the hypothalamic-pituitary-adrenal (HPA) axis response to stress than it is with any particular psychological state that is influenced by stress. Thus, the cortisol response to stressful life events might represent an intermediate phenotype between the 5-HTTLPR and psychological disorder.

Alterations in HPA axis activity are present in subgroups of a broad array of psychological disorders (14). For example, hypercortisolemia is characteristic of certain depressed patients, which has led to speculation that heightened cortisol levels might actually be causal in the onset of the disorder (15). The serotonin system is ideally positioned to moderate cortisol reactivity to stressors via its ability to influence neural activity at suprahypothalamic levels as well as at each level of the hypothalamic-pituitary-adrenal axis (16,17). In particular, serotonin fibers activate the hypothalamic corticotrophin-releasing factor containing neurons (18,19) that initiate the hormonal cascade leading to cortisol release. Therefore, variation in serotonin system functioning, for which the 5-HTTLPR is a marker, might be an important link between exposure to stressful life events and the altered HPA axis activity seen in some patient subgroups. However, studying the relationship between the 5-HTTLPR and cortisol reactivity in clinical samples is complicated by the effects of the disease state on both the HPA axis and the serotonin system (20).

Therefore, in this study, the goal was to determine whether the 5-HTTLPR is associated with cortisol responses to laboratory-based psychosocial stressors in a healthy adult sample. An association of the 5-HTTLPR with cortisol reactivity to psychosocial stress would suggest that one means by which the 5-HTTLPR influences mental health and well-being might be via differential cortisol exposure from experiencing stress.

Methods and Materials

Participants

Participants responded to a poster offering \$120 compensation for participation in the study. Prospective participants were screened during a telephone interview, and those who were currently being treated by a mental health professional, had mental or physical health problems (including post-traumatic

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stress disorder), or were using mental-health related medications (e.g., Prozac) or other medications affecting cardiovascular or endocrine function were excluded. In addition, pregnant or lactating women were excluded. All procedures were approved by the Institutional Review Board from the University of California, Los Angeles. The final sample consisted of 185 participants (39% male; 61% female; age range: 18–35 years). Because participants were affiliated with the University as students, employees, or both, the sample reflects these demographics and was 37% Asian-American, 22% European-American, 16% Latino, 23% of “mixed” ethnicity, and 2% African American (these last three groups are designated “other” in the analyses to be reported).

Trier Social Stress Test

Participants reported to the University’s Clinical Research Center in the mid to late afternoon to control for diurnal variation in cortisol (21). After arrival, participants were further screened for: 1) blood pressure <140/90 mm Hg, 2) a resting pulse between 60 and 100, 3) normal lung auscultation, and 4) a normal cardiac examination (no evidence of congestive heart failure or arrhythmia). Participants then provided a baseline saliva sample from which cortisol concentrations were measured. The nurse then inserted an indwelling catheter and performed a blood draw to assay gonadal hormone and peptide levels (data not reported here). Blood pressure and heart rate were assessed every 3 min via a Critikon Dinamap Vital Signs Monitor Model 1846SX (data not reported here). After 30 min of screening for medical problems, the second saliva sample was taken. At this time, participants completed the Positive and Negative Affect Schedule (PANAS) (22), which served as a baseline for self-reported response to the stress task. Cortisol samples at 60 and 30 min before the onset of the Trier Social Stress Test (TSST) were averaged to form the baseline measure.

Each participant then took part in the TSST, a widely used laboratory stress challenge known to elicit autonomic and HPA axis stress responses (23). Participants performed the TSST in front of a video camera. The experimenter sat off to the side and out of direct view of the participant and did not give any verbal or nonverbal indications of positive or negative reactions to the participant’s performance.

Participants were given 5 min to prepare a 5-min speech on why they would be a good administrative assistant, a popular campus job for students and employees. They then delivered the speech under one of three audience conditions. In the negative audience condition, the participant delivered his/her speech to two members of an evaluative panel who gave non-verbal indications of frustration over the quality of the speech. They displayed non-verbal signs of boredom and exchanged glances with each other that communicated mutual negative assessments. This manipulation represents a somewhat stronger version of the standardized audience condition for the TSST. In the positive audience condition, the two audience members leaned forward, smiled, and gave non-verbal indications of approval. They occasionally exchanged glances with each other that communicated mutual positive assessments, and when not explicitly communicating positive assessments, their demeanor communicated interest in what the participant was saying. The two audience conditions mirrored each other precisely in terms of the timing and type of feedback, with the exception that the nonverbal feedback was positive in one condition and negative in the other. All panels included one man and one woman, and measures were taken to ensure the participant and audience

members were unacquainted. The no audience control condition did not include an audience.

After the speech, participants performed challenging mental arithmetic tasks for 5 min that required counting backwards by 7 and by 13 from 2935 out loud, during which time they were urged by the experimenter to try to go faster. For the participants in the two audience conditions, these math problems were completed in front of the audience as well.

Twenty minutes after the commencement of the TSST, participants provided a third saliva sample. This time lag falls within the recommended window for observing stress-related increases in cortisol (23). Participants then rated their affective state on the PANAS. At 40 min after the onset of the TSST, the fourth cortisol sample was taken. A 35-min recovery period then took place after which the fifth (recovery) cortisol sample was taken. This time lag is typically associated with significant declines in cortisol levels from peak stress, although not always full return to baseline (24). At the conclusion of the experiment, the participant was debriefed and then dismissed.

Genotyping

The DNA was collected from saliva with Oragene kits (DNA Genotek, Ottawa, Ontario, Canada) and extracted according to the manufacturer’s recommendations. The 5-HTTLPR was genotyped as described previously (5) with a protocol modified from (4). Briefly, the forward primer was 5′-GGC GTT GCC GCT CTG AAT GC-3′ (labeled with 6-carboxyfluorescein fluorophore), and the reverse primer was 5′-GAG GGA CTG AGC TGG ACA ACC AC-3′, which yielded 486 bp (short) and 529 bp (long) fragments. Polymerase chain reaction (PCR) was performed in a total volume of 25 μ L, containing 100 ng of DNA, 160 nmol/L of each primer, 1 mmol/L Tris-hydrochloride (pH 8.3), 5 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, 2% dimethyl sulfoxide (v/v), 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California), 200 μ mol/L of deoxyadenosine triphosphate, deoxycytidine triphosphate, thymidine triphosphate, and 100 μ mol/L of deoxyguanosine triphosphate (dGTP), and 7-deaza-2′-dGTP. After an initial denaturation at 94°C for 5 min, 35 cycles of denaturation (94°C for 30 sec), annealing (63°C for 30 sec), and extension (72°C for 1 min) were performed, followed by a final extension at 72°C for 20 min. The PCR products were electrophoresed on an ABI 3730 DNA analyzer (Applied Biosystems) with a Mapmaker size standard (Bioventures, Murfreesboro, Tennessee). Data collection and analysis used GeneScan and Genotyper software (Applied Biosystems).

Salivary Cortisol Assay Procedures

Saliva was collected with the passive drool method into a 2.0 mL Corning cryovial (Corning, Corning, New York), which was immediately placed on ice and then transferred to a –20°C freezer for storage. The concentrations of cortisol within these samples were quantified by Salimetrics LLC (State College, Pennsylvania). The radioimmunoassay was run in duplicate with 25- μ L samples with the HS-cortisol High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics LLC). The HS-Cortisol Assay allows for robust results when the saliva samples have a pH within the range of 3.5–9.0. All samples were within this pH range. The inter- and intraassay coefficients of variation were 6.1% and 5.0%, respectively.

Statistical Analyses

Tests of Hardy–Weinberg equilibrium for the entire sample and each ethnic grouping were conducted with the software

program Haploview v3.32 (<http://www.broad.mit.edu/mpg/haploview/>) (25). Remaining statistical analyses were performed with SPSS 14.0 (Chicago, Illinois). Chi-square tests were used to assess allele distributions (2n). For assessment of cortisol responses to the TSST, repeated measures analyses of variance (ANOVAs) were used with cortisol sample time point as a within subjects variable and genotype as a between subjects variable. Greenhouse-Geisser corrections for repeated measures were used due to the violation of sphericity assumptions. For significant results, planned pair-wise comparisons used the Bonferroni correction for multiple comparisons, unless otherwise noted. When ethnicity was used as a covariate, two dummy variables were created (i.e., East Asian = 1, all others = 0; and European-American = 1, all others = 0). Due to the skewed distributions, cortisol values were log transformed before analysis. All statistical tests were two-tailed with α set to $p < .05$.

Results

One participant could not be genotyped, and two participants did not have the complete time course of cortisol samples, leaving a sample of 182 participants. Across the three experimental conditions, there were no differences in the distribution of gender [$\chi^2(2, n = 182) = .05, p = .98$], ethnicity [$\chi^2(4, n = 182) = 1.69, p = .79$], or 5-HTTLPR genotype [$\chi^2(4, n = 182) = 2.19, p = .70$].

As expected (26), 5-HTTLPR alleles were differentially distributed as a function of ethnic grouping [$\chi^2(2, n = 364) = 12.42, p = .002$]. Among participants of East Asian ancestry, the 5-HTTLPR allele frequency was 70% for the short allele and 30% for the long allele. This allele distribution was significantly different from those declaring European ancestry [Short allele: 47.6%; $\chi^2(1, n = 222) = 11.02, p = .001$] or Other ancestry [Short allele: 54.1%; $\chi^2(1, n = 286) = 7.65, p = .006$]. There were no differences in allele distribution between the group with European ancestry and the group with "Other" ancestry [$\chi^2(1, n = 228) = .9, p = .34$]. Hardy-Weinberg equilibrium calculations showed no significant deviation from equilibrium for each ethnic grouping (all p values $> .4$), as was the case for the calculation with the entire sample ($p = .19$).

Preliminary Analyses

Across all experimental conditions, cortisol levels showed a significant rise after the onset of the public speaking task and then recovered close to baseline [$F(3,546) = 56.1, p < .001, \eta^2 = .24$]. There was a significant interaction between audience condition and time [$F(4.29,386.08) = 6.31, p < .001, \eta^2 = .066$].

Although there were significant increases in cortisol levels in each of the experimental conditions [the no audience condition $F(1.96,125.27) = 12.94, p < .001, \eta^2 = .17$; the supportive evaluative audience condition $F(1.76,102.3) = 27.95, p < .001, \eta^2 = .33$; and the negative evaluative audience condition $F(2.46,142.72) = 22.99, p < .001, \eta^2 = .28$], post-test comparisons revealed that both the positive evaluative audience ($p < .001$) and the negative evaluative audience ($p = .045$) were significantly different from the no audience condition. The two evaluative audience conditions were not significantly different from each other ($p = .175$), prompting analysis of the two evaluative audience conditions together.

With respect to ethnicity, there were no significant ethnic differences in cortisol responses in the no audience condition [$F(3.91,121.07) = .30, p = .88$] or in the combined evaluative audience conditions [$F(4.31,248) = .48, p = .82$]. When each evaluative audience condition was assessed separately there were also no significant ethnic differences in cortisol response [supportive evaluative audience condition $F(3.51,98.4) = .11, p = .97$; negative evaluative audience condition $F(4.85,135.76) = .7, p = .62$]. Likewise, there were no significant gender differences in response to the stressor in the no audience condition [$F(1.95,123.07) = .25, p = .77$] or in the combined evaluative audience conditions [$F(2.19,253.95) = 1.13, p = .33$]. Similarly, there were no significant effects of gender on cortisol reactivity in the supportive evaluative audience condition [$F(1.77,101.11) = .62, p = .52$] and the negative evaluative audience condition [$F(2.47,140.93) = .66, p = .55$].

The 5-HTTLPR and Cortisol Response to Psychosocial Stress

To assess genetic moderation of cortisol reactivity, we first assessed the relationship between the 5-HTTLPR and reactivity to social evaluation (i.e., positive and negative audience conditions combined). The analyses revealed a significant relationship between the 5-HTTLPR and cortisol reactivity [$F(4.38,251.68) = 2.78, p = .024, \eta^2 = .046$]. Specifically, as Figure 1 shows, the short/short individuals exhibited the strongest cortisol responses to social evaluation. These results were unchanged when gender and ethnicity were entered as covariates [$F(4.37,244.24) = 2.67, p = .029, \eta^2 = .051$].

With respect to the specific time points, one-way ANOVAs revealed no differences in cortisol levels by allele distribution at baseline [$F(2,115) = .57, p = .57$], 20 min poststressor onset [$F(2,115) = 1.05, p = .35$], or recovery [$F(2,115) = .54, p = .59$], but there were significant differences in cortisol levels at 40 min

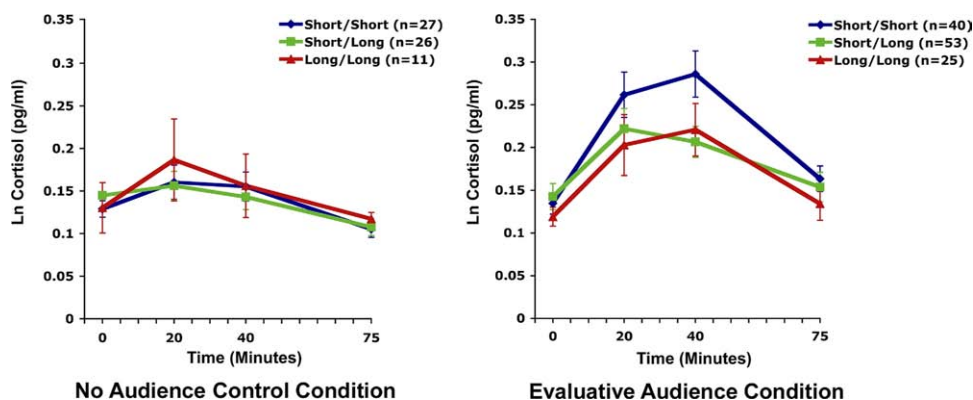


Figure 1. Mean (\pm SEM) cortisol response to the Trier Social Stress Test as a function of the serotonin transporter polymorphism in the no audience condition and the evaluative audience conditions. Ordinate values are the natural logarithm (Ln) of the raw cortisol values.

poststressor onset [$F(2,115) = 3.34, p = .04, \eta^2 = .06$] with the short/short individuals having the highest cortisol levels.

We next conducted follow-up internal analyses to examine the relationship between 5-HTTLPR genotype and cortisol response in each audience condition. In the no audience condition, there was no relationship between the 5-HTTLPR and cortisol response [$F(3.88,118.44) = .68, p = .6$]. In the supportive evaluative audience condition, the relationship between the 5-HTTLPR and cortisol response was not significant [$F(3.59,100.46) = 1.41, p = .24$]. However, in the negative evaluative audience condition, there was a significant relationship between the 5-HTTLPR and the cortisol response [$F(4.94,138.17) = 3.37, p = .007, \eta^2 = .28$]. The short/short individuals had the strongest response and the long/long individuals, the least response ($p = .042$, least significant difference), with the short/long individuals falling in between. These results remained significant when ethnicity and gender were entered as covariates [$F(5.1, 129.98) = 3.42, p = .006, \eta^2 = .11$].

To ensure that these significant results were not due to population stratification, the relationship between the 5-HTTLPR and cortisol response was examined separately for each ethnic category. Although the comparisons with this subdivided sample were not significant due to reduced power, the qualitative pattern of cortisol response was the same in each ethnic group, with a graded genotype-dependent decrease in cortisol response, with the short/short individuals being the highest. Thus, among those of European descent (mean = .25, SD = .08), those of East Asian descent (mean = .26, SD = .13), and those of Other ancestry (mean = .37, SD = .21), the mean peak cortisol response of the short/short individuals was greater than that in the short/long individuals (European ancestry: mean = .2, SD = .11; East Asian ancestry, mean = .19, SD = .08; Other ancestry, mean = .21, SD = .13). In turn, the short/long heterozygotes had higher peak cortisol responses than the long/long individuals in both the European ancestry (mean = .19, SD = .13) and Other ancestry (mean = .13, SD = .13) group.

Consistent with findings from a meta-analysis of laboratory stress studies (24), self-reported negative affect did not correlate with the changes in cortisol. Thus, changes in negative affect between the pre- and poststressor ratings were not significantly different among the three audience conditions [$F(2,176) = .23, p = .8$]. There was also no relationship between the 5-HTTLPR and changes in negative affect in any of the three audience conditions [no audience: $F(2,58) = .096, p = .91$; positive audience: $F(2,53) = 1.06, p = .35$; negative audience $F(2,53) = .41, p = .67$].

Discussion

The results of this investigation reveal a significant relationship between the 5-HTTLPR and cortisol reactivity to laboratory stressors. Those with the short/short genotype had the greatest increase in cortisol levels, followed by those with the short/long genotype, followed by those with the long/long genotype. In addition, the relationship between genotype and cortisol reactivity was strongest when individuals were exposed to a negative evaluative audience, indicating that the short/short genotype seems to confer sensitivity to social threat.

This greater sensitivity in the short allele homozygotes is consistent with other recent studies using stress paradigms similar to the one used here. In a sample of girls, some who were at familial risk for depression, short/short individuals had the greatest cortisol response to psychosocial stress (27). Similarly, in an adult sample that included healthy participants, depressed

patients, and individuals at familial risk for depression, women with the short/short genotype had the greatest cortisol response to psychosocial stress (28). Thus, across studies, a consistent picture is emerging indicating that the short/short genotype of the 5-HTTLPR is associated with higher cortisol response to psychosocial stress. The consistency in the direction of effects across these studies indicates that the study of intermediate phenotypes such as cortisol reactivity might be of great utility in identifying genetic associations, relative to studies of broader phenotypes such as psychiatric diagnoses.

The presence or absence of an evaluative audience in the present study also helps to provide psychological insight into the cognitive processes influenced by the 5-HTTLPR. According to a recent meta-analysis (24), two principal psychological factors mediate the effect of psychosocial stress on cortisol response: a lack of control, and threat to self-esteem or status (social evaluative threat). Compared with the no audience condition, both of these factors were accentuated in the evaluative audience conditions, especially in the negative evaluation condition. Lack of control associated with the inability to avoid social evaluation might be akin to the uncontrollable stressors used in animal studies, which show greater activation of the serotonin system compared with controllable stressors (29).

The association of the 5-HTTLPR and cortisol reactivity seen in the present research is consistent with studies that have focused on amygdala reactivity as an intermediate phenotype associated with the 5-HTTLPR. In a small study comparing the neural correlates of a speech delivered in the presence or absence of an audience, short allele carriers were found to have greater amygdala activation than long allele homozygotes when an audience was present (30). Due to the confines imposed by the scanning environment, the participants in this study could not see the audience, indicating that the mere awareness of being evaluated, irrespective of the reaction of the audience, can lead to differential amygdala activation as a function of 5-HTTLPR genotype. With respect to the present study, although the pressure of social evaluation seems to be a main activator of the cortisol response, the significant association of cortisol reactivity with the 5-HTTLPR in the negative evaluative audience condition might be due to the greater sensitivity of short/short individuals to the threatening, critical, and disapproving feedback in this condition. This would be consistent with functional neuroimaging studies that have shown greater amygdala activity in short allele carriers when viewing threatening faces (31; meta-analysis [32]). As stimulation of the amygdala can trigger HPA axis activation (33) and lesions to the amygdala prevent serotonin neurons from activating the HPA axis (34), the amygdala is likely to be a critical site mediating the greater cortisol reactivity of short/short individuals to the threatening social cues in the negative audience condition in the present study and potentially the threat of social evaluation more generally.

In addition to a role in activating the HPA axis, the serotonin system can also influence feedback inhibition of the HPA axis, particularly via the hippocampus (17). The time point showing the greatest differentiation between short/short individuals and the other groups was 40 min after stressor onset (Figure 1), reflecting persisting activation of the HPA axis in short/short individuals relative to those with the long allele. Thus, the heightened cortisol response in these individuals might not only be due to greater HPA axis activation but also to impaired feedback inhibition. This would be consistent with findings in the serotonin transporter knockout mouse (35) as well as human data showing that serotonin transporter inhibitor treatment influ-

ences feedback inhibition (36). Further studies will be needed to address this issue definitively.

Limitations

One limitation of this study is the heterogeneous ethnic composition of the sample, which raises concerns over potential population stratification artifacts (37). However, in spite of the differences in allele frequency between ethnic groups, the qualitative relationship between the 5-HTTLPR and cortisol responses was similar. That the 5-HTTLPR seems to function similarly across ethnic groups is further evidenced by a recent study in an exclusively Korean sample showing that carriers of the short allele were at higher risk for depression when exposed to stressful life events than long allele homozygotes (38), which is akin to the original finding of Caspi *et al.* (6) in a Caucasian sample. This is consistent with a meta-analysis of replicated disease–gene association studies that showed the vast majority of associations are similar across ethnic groups (39).

The specific focus on the 5-HTTLPR in this study assumes that it is a functional polymorphism affecting serotonin transporter gene expression. Although expression assays with cells from the periphery indicate this to be the case (4), data from neural tissue have provided less clarity (40,41). Thus, greater consistency across studies could potentially be obtained with more detailed assessment of variation in the vicinity of the 5-HTTLPR (e.g., rs25531) (42) or the *SLC6A4* locus in general (43).

It should be noted that the serotonin system can influence the HPA axis at the hypothalamic, pituitary, and adrenal levels (16). Thus, with only cortisol measurements, which reflect the output of the adrenal gland, it is not possible to delineate the specific level at which the 5-HTTLPR is altering responsivity to social stress. Although the most parsimonious explanation is that the 5-HTTLPR is affecting HPA axis activity via influence at the hypothalamic level, it could also be associated with modulation of adrenocorticotrophic hormone release from the pituitary or cortisol release from the adrenal gland.

The findings reported here are based on reactions to laboratory stressors rather than naturalistic stressors. Although this methodological choice confers some potential disadvantages, it might have benefits as well. The ability to experimentally control the magnitude and duration of the stressor might reduce some of the variance associated with measurement of exposure to stressors in daily life (44).

Because this sample excluded individuals with any current or past mental health problems, it precludes making direct applications to clinical disorders for which disease-related processes might influence responsivity of the HPA axis. Nonetheless, the fact that the 5-HTTLPR is associated with cortisol reactivity in this nonclinical sample broadens the significance of the 5-HTTLPR's relation to cortisol reactivity, suggesting a pervasive risk factor for a broad array of stress-related disorders.

Conclusions

We report a significant relationship between the 5-HTTLPR and cortisol response to laboratory stressors. The heightened cortisol reactivity of short/short individuals, especially in the negative audience condition, indicates that these individuals are especially sensitive to social evaluation, and short/short individuals might have greater vulnerability to stress-related mental health outcomes as a result.

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