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Serotonin transporter polymorphism predicts waking cortisol in young girls

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Summary Major Depressive Disorder (MDD) is one of the most prevalent and costly of all psychiatric disorders. The hypothalamic pituitary adrenal (HPA)-axis, which regulates the hormonal response to stress, has been found to be disrupted in depression. HPA dysregulation may represent an important risk factor for depression. To examine a possible genetic underpinning of this risk factor without the confound of current or lifetime depression, we genotyped 84 never-disordered young girls, over a third of whom were at elevated risk for depression, to assess the association between a polymorphism in the promoter region of the serotonin transporter (5-HTT) gene and diurnal variation in HPA-axis activity. This 5-HTT-linked polymorphic region (5-HTTLPR) has been previously found to interact with stress to increase risk for depression. We found 5-HTTLPR to be significantly associated with diurnal cortisol levels: girls who were homozygous for the short-allele had higher levels of waking (but not afternoon or evening) cortisol than did their long-allele counterparts. This finding suggests that genetic susceptibility to HPA-axis dysregulation, especially apparent in levels of waking cortisol, is detectable in individuals as young as 9 years of age.

1. Introduction

Major Depressive Disorder (MDD) is among the most prevalent and costly of all psychiatric disorders. About 16% of the general population will experience an episode of MDD in their lifetimes, and up to two-thirds of these individuals will experience recurrent episodes (Eaton et al., 2008; Kessler et al., 2003). Given the extraordinary societal costs and the personal burden that are associated with this disorder (e.g., Greenberg et al., 2003), it is critical that we identify and elucidate factors that place individuals at elevated risk for the development of MDD.

In this context, a number of investigators have examined the serotonin system in depression (see Thase, 2008, for a review). Most recently, spurred in part by the effectiveness of selective serotonin reuptake inhibitors (SSRIs) in the treatment of depression, researchers have begun to examine the role of the genetics of the serotonin system in placing individuals at elevated risk for this disorder (Levinson, 2006). Specifically, several investigators have found the serotonin transporter gene (5-HTT) linked polymorphic region (5-HTTLPR) to interact with major life stress to predict the

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onset of MDD (e.g., Caspi et al., 2003; Kendler et al., 2005); when exposed to stress, individuals who are homozygous for the short-allele (SS individuals) are more likely to develop depression than are individuals with one or two long alleles (SL or LL individuals). The involvement of life stress in this interaction is consistent with a large literature implicating stress in the onset of depression (Monroe et al., 2008) and underscores the importance of investigating stress reactivity in people who are vulnerable to the development of this disorder. In fact, depressed individuals have been found to be characterized by dysfunctional activity of the hypothalamic pituitary adrenal (HPA)-axis, which regulates hormonal responses to stress. More specifically, depressed persons exhibit elevated levels of cortisol (Gillespie and Nemeroff, 2005). In fact, hypercortisolemia is associated not only with increased severity of depression (Whiteford et al., 1987), but with hippocampal changes and damage (McEwen, 1999; Sapolsky, 2001) and with increased risk for cardiovascular disorders (Weber-Hamann et al., 2002).

Recently, Gotlib et al. (2008) found that the same polymorphism in 5-HTTLPR that interacts with life stress to predict the onset of depression is associated with increased cortisol response to an acute laboratory stressor. It is important to note that while the HPA-axis produces cortisol in response to acute stressors, HPA-axis activity also follows a diurnal rhythm, with cortisol levels typically rising with morning waking and declining throughout the day (Posener et al., 1996; Born et al., 1999). Anomalous diurnal patterns of cortisol secretion may indicate systemic dysregulation in HPA-axis feedback; indeed, investigators have documented elevated levels of waking cortisol in individuals diagnosed with MDD (Young, 2004; Bhagwagar et al., 2005). Perhaps not surprisingly given the association of serotonin with depression (cf. Thase, 2008), evidence from both human and animal studies indicates that the regulation of HPA-axis function involves the serotonergic system (e.g., Porter et al., 2004; Linthorst, 2005). Although Gotlib et al. (2008) recently demonstrated that the 5-HTTLPR polymorphism is associated with cortisol response to an acute stressor, it is not known whether the effects of the 5-HTTLPR polymorphism are confined to stress reactivity cortisol, or whether this polymorphism would also be associated with differential systemic HPA-axis function. The present study was designed to examine whether 5-HTTLPR polymorphisms are related to diurnal cortisol production.

We predicted that homozygous short-allele carriers would be characterized by higher levels of diurnal cortisol than would heterozygous participants, with homozygous long-allele carriers producing the lowest levels of diurnal cortisol. Moreover, given recent findings that depression is associated with increased levels of waking cortisol in particular (Bhagwagar et al., 2005), we predicted that the genetic effects on cortisol production would be strongest during the morning, immediately after waking. Finally, because we wanted both to examine the relation between 5-HTTLPR polymorphisms and diurnal cortisol without the confound of current or past depression and to have a sufficient number of homozygous and heterozygous short- and long-allele participants, we assayed diurnal cortisol from two groups of participants with no current or past episodes of depression: daughters of mothers who had no history of depression, and daughters of mothers who had a history of recurrent depression during their daughters' lifetime. We examined only female participants both because of the higher prevalence of MDD in females than in males (Kessler et al., 2003) and to increase the homogeneity of the sample.

2. Method

2.1. Participants

Participants were 84 girls between the ages 9 and 14 who had no current or past DSM-IV Axis I disorder. Fifty-three of these girls were daughters of mothers who also had no current or past Axis I disorder (low risk for depression), and 31 were high-risk daughters of mothers who had a history of recurrent episodes of Major Depressive Disorder during their daughters' lifetime (high risk for depression); all daughters in the study came from different families. Participants were recruited through internet and print advertisements in the local community or through the Department of Psychiatry and Behavioral Sciences at Stanford University. The mothers' responses to a telephone interview provided initial selection information. This phone screen established that both mothers and daughters were fluent in English and that daughters were between 9 and 14 years of age. The telephone interview was also used to identify mothers who were likely either to have no psychiatric history or to meet criteria for recurrent depression during their daughter's lifetime, and daughters who were likely to have no past or current psychiatric disorder. Those mother and daughter pairs who were considered likely to be eligible for participation were invited to come to the laboratory for more extensive interviews.

2.2. Assessment of depression and psychopathology

At the interview session, trained interviewers assessed the diagnostic status of daughters by administering the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (K-SADS-PL; Kaufman et al., 1997) separately to the daughters and to their mothers (about the daughters). The full interview, assessing current and lifetime diagnoses for affective, psychotic, anxiety, behavioral, substance abuse, and eating disorders was administered. Due to time constraints and the fact that all our participants were female, the Tic Disorders and ADHD screeners were not administered (both of these disorders are more prevalent in boys: Gadow et al., 2002). The K-SADS-PL has been shown to generate reliable and valid child psychiatric diagnoses (Kaufman et al., 1997). A different interviewer administered the Structured Clinical Interview for the DSM-IV (SCID; First et al., 1997) to the mothers. The SCID has demonstrated good reliability for the majority of the disorders covered in the interview (Skre et al., 1991; Williams et al., 1992). Both K-SADS-PL and SCID-I interviewers had previous experience with administering structured clinical interviews and were trained specifically to administer these interviews. To assess interrater reliability, an independent trained rater who was blind to group membership evaluated 30% of the SCID and K-SAD-PL interviews by randomly selecting audiotapes of equal numbers of at-risk and control pairs. In all cases, diagnoses of former depressive episodes in

mothers, no history of depressive episodes in mothers, and absence of any current or previous Axis-I disorder in the girls matched the diagnosis made by the original interviewer, $\kappa = 1.00$, indicating excellent interrater reliability.

Eligible participants gave a saliva sample for genotyping (see below), and daughters completed the 10-item version of the Children's Depression Inventory (CDI; Kovacs, 1985), a self-report measure of depressive symptoms for children between the ages of 8 and 17. Mothers completed the Beck Depression Inventory-II (BDI; Beck et al., 1996), a frequently used 21-item self-report measure of depressive symptom severity.

2.3. Diurnal cortisol collection

Within 2 weeks of the initial assessment, daughters and mothers were given salivette kits (Sarstedt, Germany) for at-home measurement of cortisol. Both mothers and daughters completed 2 consecutive days of measurements, with four measurements per day: at awakening, 30 min post-awakening, mid-afternoon, and 30 min before bedtime. Participants were instructed to place salivettes in the freezer immediately after sampling and were given bottles whose caps (unbeknownst to the participants) tracked the times at which the bottles were opened. Saliva samples were stored in a freezer by participants until they completed all the measurements. Samples were then transferred to a −20 °F freezer in the Stanford University General Clinical Research Center, where they were kept until radioimmunoassay. Samples were assayed together to control for interassay error, with control samples included to evaluate variability. A minimum of 0.2 ml of liquid saliva was collected by absorption into a small cotton roll and expressed through a plastic tube into a sterile vial. Cortisol levels were assayed by luminescence immunoassay reagents using a commercial kit from Immuno-Biological Laboratories Inc. (Hamburg, Germany). The assay sensitivity was set at 0.015 mg/dl. The intraassay variation on three saliva pools of the low, medium, and high controls were averaged 2.78%, 10.45%, and 4.79%, respectively. The mean values of the low, medium, and high controls were .054, .228, and .863 mg/dL, respectively. The interassay coefficients of the variations of the low, medium, and high controls were 10.9%, 10.5%, and 5.5%, respectively.

2.4. Genotyping

To genotype the daughters, saliva was collected using the Oragene Kit (DNA Genotek Inc., Ottawa, Ontario, Canada), an all-in-one system for the collection, preservation, transportation, and purification of DNA from saliva. DNA extracted by this method is of high quality and allows for a high success rate of genotyping (26); indeed, the DNA yield in our laboratory is 20 μg and higher. Oligonucleotide primers flanking the 5-HTT-linked polymorphic region (27) and corresponding to the nucleotide positions −1416 to −1397 (stpr5, 5′-GGC GTT GCC GCT CTG AAT GC) and −910 to −888 (stpr3, 5′-GAG GGA CTG AGC TGG ACA ACC AC) of the 5-HTT gene 5′-flanking regulatory region were used to generate 484 bp or 528 bp fragments. The PCR products were electrophoresed through 5% Polyacrylamide gel (Acrylamide/bis-Acrylamide ratio 19:1) at 60 V for 60 min.

Table 1 Characteristics of participants by risk group and by genotype group, and genotype frequencies for the low- and high-risk participants.

	Low-risk	High-risk
# of participants	53	31
Age in years	12.53 (1.53)	12.69 (1.52)
CDI	1.2 (1.4)	3.0 (2.9)

	LL	SL	SS
# of participants	19	46	19
Age in years	12.62 (1.40)	12.46 (1.64)	12.87 (1.34)
CDI	2.61 (2.8)	1.78 (2.4)	1.44 (1.2)

	LL	LS	SS	Total
Low-risk	15	26	12	53
High-risk	4	20	7	31
Total	19	46	19	84

Note: Standard deviations are shown in parentheses; CDI = Child Depression Inventory; LL = two copies of the long-allele of 5-HTTLPR; LS = one copy of the long-allele and one copy of the short-allele of 5-HTTLPR; SS = two copies of the short-allele of 5-HTTLPR.

3. Results

3.1. Participant characteristics

Demographic and clinical characteristics of the low- and high-risk girls are presented in Table 1. The two groups of girls did not differ with respect to either age, $t(82) < 1$, or the proportion of girls who were post-menarcheal, $\chi^2(1) = 2.9$, both $ps > .05$. Although the high-risk girls had slightly but significantly higher scores on the CDI than did the low-risk girls, $t(79) = 3.2$, $p < .05$, it is important to note both that scores of both groups of girls are well below the minimum score of 8 recommended as a cut-off for possible depression (Kovacs, 1985), and that CDI scores were not significantly correlated with any measure of diurnal cortisol. 5-HTTLPR genotype frequencies for the low- and high-risk participants are also presented in Table 1. The two groups of girls did not differ with respect to genotype frequencies, $\chi^2(2) = 2.90$, $p > .05$. Furthermore, in the full sample of girls, the three 5-HTTLPR genotype groups (SS, SL, LL) did not differ significantly with respect to age, $F(2,82) = 0.32$, CDI score, $F(2,80) = 1.30$, or proportion of girls who were post-menarcheal, $\chi^2(2) < 1$, all $ps > .05$.[1]

3.2. Cortisol

Several cortisol samples were found to positively skew the data, despite logarithmic and other transformations. Conse-

[1] To further examine the possible effects of pubertal development, Tanner scores were obtained for participants. There were no significant differences in Tanner hair or breast stage as a function of either genotype (hair: $\chi^2(8) = 6.67$; breast: $\chi^2(8) = 7.71$, both $ps > .05$) or risk group (hair: $\chi^2(4) = 3.58$; breast: $\chi^2(4) = 1.81$, both $ps > .05$). Moreover, neither Tanner hair stage nor Tanner breast stage significantly predicted AUCg levels of diurnal cortisol, $F(4,72) = 1.51$ and $F(4,74) = 2.04$, respectively, both $ps > .05$.

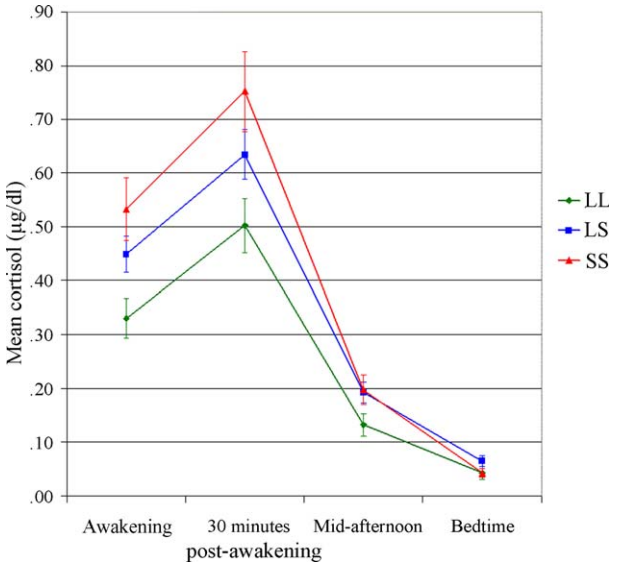

Figure 1 Diurnal cortisol as a function of 5-HTTLPR genotype. *Note*: Diurnal cortisol measured at awakening, 30 min post-awakening, mid-afternoon, and 30 min before bedtime.

quently, we winsorized values 2 standard deviations above the mean to the 2-standard deviation level based on methods described by Tukey (1977). Values for each collection time were averaged across the 2 days to calculate a more reliable mean at each time point, a procedure used in previous studies of cortisol in adolescent depression (e.g., Goodyer et al., 2000). Importantly, collection times did not differ as a function of either risk group, all $t(59) < 1.58$ for each time point, or genotype group, all $F(2,60) < 3.05$ for each time point, all $ps > .05$.

A two-way (risk group by genotype group) analysis of covariance (ANCOVA) conducted on diurnal cortisol area under the curve with respect to ground (AUCg; Pruessner et al., 2003) with CDI scores as a covariate yielded a significant effect only for genotype group, $F(2,70) = 3.33$, $p < .05$; neither the main effect for risk group, $F(1,70) = 2.64$, nor the interaction of genotype group and risk group, $F(2,70) = 1.21$, was significant, both $ps > .05$. Levels of diurnal cortisol as a function of genotype group are presented in Fig. 1. Follow-up tests indicated that the LL genotype group had significantly lower AUCg cortisol than did both the SL, $t(60) = 2.34$, $p < .05$, and the SS, $t(33) = 3.17$, $p < .005$, genotype groups, which did not differ significantly from each other, $t(59) = 1.35$, $p > .05$.

Because AUCg in this analysis included four distinct cortisol collection time points, we conducted one-way (by genotype group) analyses of variance (ANOVAs) on cortisol levels at each time point. Significant main effects for genotype group were obtained at collection time 1 (awakening), $F(2,83) = 4.05$, $p < .05$, and collection time 2 (30 min post-awakening), $F(2,82) = 3.31$, $p < .05$, but not at collection times 3 (mid-afternoon), $F(2,83) = 1.93$, or 4 (30 min before bedtime), $F(2,83) = 1.35$, both $ps > .05$. Follow-up tests conducted at the first collection time indicated that the LL genotype group had significantly lower levels of cortisol than did both the SS, $t(36) = 2.93$, $p < .01$, and the SL, $t(63) = 2.07$, $p < .05$, genotype groups, which did not differ significantly from each other, $t(63) = 1.31$, $p > .05$. At the second collection time, the LL genotype group again had significantly lower levels of diurnal cortisol than did the SS genotype group, $t(35) = 2.82$, $p < .01$; no significant differences were obtained between the SL and the LL, $t(63) = 1.67$, $p > .05$, or the SS, $t(63) = 1.35$, $p > .05$, genotype groups.

4. Discussion

Polymorphisms in the serotonin transporter gene have been implicated in the risk for major depression in epidemiological studies (Caspi et al., 2003). The mechanisms by which the short-allele polymorphism confers risk, however, have not been elucidated. Recently, investigators have implicated the HPA-axis as a possible target of modulation by 5HTT gene variation (Gotlib et al., 2008). While there have been studies documenting the interaction of serotonin and the HPA-axis (Porter et al., 2004), only O'Hara et al. (2007) have examined the effect of polymorphisms in the serotonin transporter gene on diurnal HPA-axis function, and they did so in a sample of elderly individuals. To study the effect of the polymorphisms in the context of depression, we examined the contribution of both familial risk and 5-HTTLPR to levels of waking cortisol in a sample of young girls. While there is some evidence that levels of diurnal cortisol may have a heritable component (Bartels et al., 2003), the mechanisms underlying this possible inheritance are not known. The present study is the first to assess the effects of both 5-HTTLPR and familial risk for depression on diurnal HPA-axis activity in individuals as young as 9 years of age.

We found that the presence of one or two short alleles of the 5-HTTLPR polymorphism was associated with elevated levels of waking cortisol. More specifically, girls with two copies of the short-allele had significantly higher levels of waking cortisol than did homozygous long-allele girls; girls with one long- and one short-allele had levels of waking cortisol between these two groups. Interestingly, despite evidence that diurnal cortisol cycles in older adults are steeper than they are in younger adults (Ice et al., 2004), the pattern of genotype findings in this sample of young girls parallels that obtained with older adults (O'Hara et al., 2007). When both genotype and familial risk were included in the same analysis, only genotype was a significant predictor of level of diurnal cortisol. In contrast to this finding, Mannie et al. (2007) recently reported that familial risk for depression was associated with elevated levels of waking cortisol. In understanding this discrepancy, it is important to note several differences between the present investigation and Mannie et al.'s study: our participants were, on average, almost 7 years younger than Mannie et al.'s subjects, included only females, and included only participants whose parent's recurrent depression was confirmed in a structured clinical interview.

In previous work in our laboratory, we found an association between 5-HTTLPR genotype and cortisol reactivity to experimentally induced stress (Gotlib et al., 2008). The present finding that 5-HTTLPR genotype is associated with high levels of morning cortisol production suggests that 5-HTTLPR is linked to a systemic dysregulation of HPA-axis function, and is not confined to affecting a specific reaction to psychological stress. Moreover, the fact that there were no polymorphism-related differences in afternoon or evening

cortisol levels suggests that 5-HTTLPR is not associated with a overall increase in basal cortisol, but instead, plays an important role in the serotonin-mediated negative feedback system of the HPA-axis (Porter et al., 2004). Indeed, although only a subset of participants was the same in the present study and in our earlier report, levels of stress reactivity cortisol were significantly related only with the diurnal cortisol collected 30 min after waking ($r = .32$, $p < .005$); none of the other diurnal cortisol measurements correlated with stress reactivity cortisol. Thus, the novel finding that level of reactivity cortisol is correlated with level of waking cortisol is consistent with the formulation that 5-HTTLPR plays a role in HPA-axis feedback during increases in cortisol.

While the short-allele of 5-HTTLPR has been associated with increased risk for depression in epidemiological studies (Caspi et al., 2003), the mechanism by which this genotype increases risk for psychopathology is unclear. One possibility, suggested by Porter et al. (2004), is that 5-HT may play a role in the regulating the release of stress cortisol. Indeed, investigators have found that experimentally manipulating serotonin levels leads to changes in awakening cortisol response (Vielhaber et al., 2005). We found in the present study that carriers of the short-allele of 5-HTTLPR, which has been associated with decreased 5-HTT gene transcription (Heils et al., 1996) and decreased 5-HT transport (Lesch et al., 1996), have different patterns of awakening cortisol than do individuals who are homozygous for the long-allele. Furthermore, the present finding of 5-HTTLPR genotype differences specifically in awakening cortisol, as well other recent evidence linking 5-HTTLPR and cortisol output during HPA-axis activation, suggests that dysregulation of HPA-axis feedback is a viable mechanism underlying the impact of 5-HTTLPR on risk for depression. This dysregulation may be a result of the direct effect of 5-HTTLPR on HPA-axis feedback, although other mechanisms such as amygdala reactivity (Munafò et al., 2007) or amygdala—cingulate functional connectivity (Pezawas et al., 2005) could result in similar dysregulation via the putative interactions of the amygdala with the HPA-axis in animal studies (see Herman et al., 2005). Indeed, increased cortisol may in turn increase amygdala activity, further contributing to HPA-axis dysregulation (see Gold et al., 2002).

The current findings have implications for understanding the developmental nature of the neuroendocrine changes that often accompany and that may, in fact, precede and contribute to the development of depression. The results of this study suggest that there is dysfunction in 5-HT mediated HPA-axis feedback, and that it is detectable as early as childhood. Although Dahl et al. (1991) found increased cortisol secretion in depressed adolescents just before sleep onset, we found no differences in evening cortisol. It appears that evening hypercortisolemia that is present in currently depressed adolescents does not characterize high-risk children who have not yet experienced an episode of depression. Other investigators have reported that depressed adults secrete higher levels of cortisol upon awakening than do their nondepressed counterparts (Bhagwagar et al., 2005). Importantly, we observed this specific pattern of HPA-axis dysregulation in children as young as 9 years of age, none of whom had yet developed any form of Axis-I psychopathology. It is possible, therefore, that the effects of 5-HTTLPR on glucocorticoid production are important in increasing vulnerability to the onset of MDD, especially given documentation of the detrimental effects of glucocorticoids contributing to hippocampal atrophy (Sapolsky, 2001). Thus, individuals who are homozygous for the 5-HTTLPR short-allele may not only be biologically sensitive to stressful events, but may also be vulnerable to other perturbations in HPA-axis function. Future studies should attempt to identify the specific mechanisms by which 5HTT levels regulate the HPA-axis, with the goal of ultimately elucidating new possibilities for improved diagnosis, prevention, and treatment of depression.

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Conflict of interest

All other authors declare that they have no conflicts of interest.

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