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Interaction Between *5-HTTLPR* and *BDNF* Val66Met Polymorphisms on HPA Axis Reactivity in Preschoolers

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Abstract

This study examined whether the interaction between the serotonin transporter promoter region (*5-HTTLPR*) and brain-derived neurotrophic factor (*BDNF*) Val66Met polymorphisms was associated with hypothalamic-pituitary-adrenal (HPA) axis reactivity to stress. A community sample of 144 preschool-aged children was genotyped and exposed to stress-inducing laboratory tasks. Salivary cortisol was obtained at four time points during a standardized laboratory assessment before and after stressors involving separation from a parent and frustrating tasks. Children homozygous for the *short-5-HTTLPR* allele and carrying the *Met-BDNF* allele evidenced a significantly lower initial level of cortisol, followed by a positive increase in cortisol in response to the laboratory stressors. In contrast, children who were homozygous for the *short-5-HTTLPR* and the *Val-BDNF* alleles evidenced a greater decline in cortisol in response to the laboratory stressors. Findings indicated that the *BDNF* gene moderated the association between *5-HTTLPR* and children's biological stress responses, suggesting that epistatic effects play a role in individual differences in stress regulation, and possibly genetic vulnerability to stress-related disorders.

Keywords

HPA axis reactivity; cortisol; serotonin transporter gene; BDNF; stress; multilevel modeling

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Conflict of interest
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Despite the evidence for a significant genetic contribution to psychiatric diseases, attempts at identifying candidate genes have been largely unsuccessful (e.g., Munafo, Durrant, Lewis, & Flint, 2009; Risch et al., 2009). However, more consistent findings have emerged from studies examining genes associated with intermediate phenotypes of psychiatric diseases (Gottesman & Gould, 2003), such as increased amygdala activation in response to aversive stimuli (Munafo, Brown, & Hariri, 2008) and anxiety-related personality traits (Lesch et al.,1996; Munafo, Clark & Flint, 2005). Likewise, the hypothalamic-pituitary-adrenal (HPA) axis, one of the body's main biological systems mediating the neuroendocrine response to stress, has been hypothesized as an intermediate phenotype for stress-related disorders (Flint & Munafo, 2007; Hasler, Drevets, Manji, & Charney, 2004).

Abnormalities in HPA axis responses to stress have been documented in numerous stress-related disorders (Ehlert, Gaab, Heinrichs, 2001; McEwen, 2008), including depression (Burke, Davis, Otte, & Mohr, 2005; Lopez-Duran, Kovacs, & George, 2009), anxiety disorders (Bremner et al., 2003; Leyton et al., 1996), schizophrenia (Jansen et al., 1998), substance use disorders (Lovallo, Dickensheets, Myers, Thomas, & Nixon, 2000) and a number of negative health outcomes (McEwen, 2008). During acute stress, there is an increase in the secretion of cortisol, an adrenocortical steroid hormone, and in secretions from the corticotropin-releasing factor. However, chronic HPA axis activation, referred to as allostatic load, has been shown to lead to disruptions in the regulation and negative feedback of the HPA axis, resulting in adverse biological and health consequences, including immune system dysfunction, neuronal damage in the hippocampus, diabetes, hypertension and psychiatric diseases (McEwen, 2008; Meyer, Chrousos, & Gold, 2001).

Twin studies examining the heritability of HPA axis function have demonstrated a significant hereditary component in basal free cortisol levels (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003) and in response to a laboratory stressor (Federenko, Nagamine, Hellhammer, Wadhwa, & Wust, 2004). Only recently have studies begun to investigate the influence of genetic polymorphisms on HPA axis function. One gene that has received considerable attention in studies examining the genetic vulnerability to life stress, and that has been hypothesized to influence HPA axis function, is the serotonin transporter (5-HTT) gene, specifically a polymorphism in the promoter region of this gene (*5-HTTLPR*) (Caspi et al., 2003; Uher & McGuffin, 2008). Animal studies have provided evidence that 5-HTT impacts HPA activity (Li et al., 1999; Barr et al., 2004; Jiang, Wang, Luo, & Li, 2008). In addition, recent studies in humans have found that the short allele of *5-HTTLPR*, which leads to reduced transcription of 5-HTT relative to the long allele (Lesch et al., 1995), is associated with higher basal cortisol levels (Chen, Joormann, Hallmayer, & Gotlib, 2009; O'Hara et al, 2007; Wust et al., 2009) and greater cortisol reactivity (Alexander et al., 2009; Gotlib, Joormann, Minor, & Hallmayer, 2008; Jabbi et al., 2007). Some findings suggest that this relation differs by gender and/or specific features of neuroendocrine function (Jabbi et al., 2007; Wust et al., 2009); however, this remains unclear given the limited data.

In addition to 5-HTT, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, is hypothesized to influence the body's neuroendocrine response to stress, and is central to models that view neurobiological responses to stress as playing a key role in understanding the pathophysiology of stress-related disorders (Duman & Monteggia, 2006). BDNF is involved in the development of the central nervous system (CNS) and the regulation of basic neuronal function (Bath & Lee, 2006), and also protects neurons from the damaging effects of stress (Bergstrom, Jayatissa, Mork, & Wiborg, 2008). Animal research has shown that BDNF influences HPA axis activity in rats (Givalois et al., 2004; Naert, Ixart, Tapia-Arancibia, & Givalois, 2006). These findings suggest that BDNF may be involved in mechanisms underlying HPA axis function in humans.

Most attention has focused on a valine (Val) to methionine (Met) substitution at codon 66 (Val66Met) in the *BDNF* gene. The *Met-BDNF* allele is associated with reduced BDNF activity (Chen et al., 2004) and reduced hippocampal volumes (Frodl et al., 2007), and has been linked to stress-related symptoms in animals (Chen et al., 2006) and humans (Kim et al., 2007; Wichers et al., 2008) [for conflicting findings, see Lang et al., 2005; Sen et al., 2003]. In addition, two recent studies in humans have reported an association between the *BDNF* Val66Met polymorphism and HPA axis activity in adults. Schule et al. (2006) found that homozygous *Met-BDNF* carriers evidenced greater HPA axis activity. Similarly, Shalev et al. (2009) found that females with a *Met-BDNF* allele evidenced a greater rise in cortisol is response to a laboratory challenge than *Val-BDNF* homozygotes, whereas the opposite was found for males. Taken together, both *5-HTTLPR* and *BDNF* appear to be possible candidate genes underlying stress reactivity.

Importantly, 5-HTT and BDNF interact at intracellular and intercellular levels (Duman, Heninger, & Nestler, 1997). Evidence suggests that these genes have a synergistic influence on HPA axis functioning. For example, 5-HTT knockout mice bred with *BDNF* heterozygous mice evidence increased stress hormones compared to mice with knockouts in only one system (Ren-Patterson et al., 2005). In addition, two studies found that the combination of the *short-5-HTTLPR* allele and the *Met-BDNF* allele predicted depressive symptomatology in the presence of environmental adversity in children and adults (Kaufman et al., 2006; Kim et al., 2007); however, conflicting findings suggest that the *Met-BDNF* allele has a protective effect on the impact of the *short-5-HTTLPR* allele on brain morphology (Pezawas et al., 2008). Nevertheless, these findings raise the possibility that *BDNF* may interact with *5-HTTLPR* to influence HPA axis reactivity to stress, thereby rendering certain individuals more vulnerable to life stress. However, no studies have directly examined this gene-gene interaction on HPA axis function.

This study aimed to add to the limited research examining the effects of *5-HTTLPR* and *BDNF* genes on HPA axis reactivity. We were interested in advancing the understanding of epistasis as it relates to HPA axis functioning, particularly during early childhood, which appears to be a critical period in the development of the HPA axis system (Gunnar & Vazquez, 2006). We hypothesized that the interaction between *5-HTTLPR* and *BDNF* Val66Met would be associated with individual differences in children's cortisol responses to laboratory stressors. Specifically, we hypothesized that children homozygous for the *short-5-HTTLPR* allele and carrying the *Met-BDNF* allele would exhibit a greater increase in cortisol reactivity to laboratory stressors than other genotype groups, consistent with findings from animal (Ren-Patterson et al., 2005) and human studies (Kaufman et al., 2006; Kim et al., 2007).

No prior research has examined the influence of genetic polymorphisms on HPA axis functioning in young children. Understanding the genetic factors involved in the early regulation and development of the HPA axis is particularly important as the stress system affects the development, organization, and plasticity of many neural systems (Gunnar & Quevedo, 2007), and provides a means to understanding early-emerging developmental trajectories of adjustment and maladjustment.

Method

Participants

The sample was obtained from a consecutive series of 166 children who were recruited from a larger community sample participating in a study on temperament and risk for depression (N = 559). Of the 166 children asked to participate in the cortisol and genetic assessment, 156 children completed both the laboratory salivary cortisol samplings and provided buccal swabs for genetic analysis. Of the 156 children, 12 children were excluded, yielding a final sample

of 144 children (50% female): one child whose *BDNF* genotyping yielded discrepant results (described below) and 11 children of non-Caucasian or unknown ethnicity were excluded. We chose to restrict our sample to an ethnically homogenous sample (i.e., Caucasian only) because ethnicity-related differences in allelic frequencies have been reported for these genes (Kim et al., 2007; Shimizu, Hashimoto, & Iyo, 2004). Given that the optimal approach to addressing the effects of ethnicity-related or other population-related genetic differences are controversial, we chose a conservative approach to limit sources of bias (Hutchison, Stallings, McGeary, & Bryan, 2004; Wacholder, Rothman, & Caporaso, 2002).[1]

The sample was recruited using a commercial mailing list. Children between the ages of 3 and 4 years, with no significant medical or developmental disabilities, and who lived with at least one biological parent were eligible. The mean age of the children from the subsample was 43.2 months (SD = 2.4). Most of the participants came from middle class, two-parent (98.1%) families. Children were of average cognitive ability as indexed by the Peabody Picture Vocabulary Test (Dunn & Dunn, 1997; M = 105.04, SD = 14.08) and the Expressive One-Word Picture Vocabulary Test (Brownell, 2000; M = 100.48, SD = 12.47). The Committees on Research Involving Human Subjects at Stony Brook University approved and oversaw the study. Families were compensated financially.

Procedures

The child and one parent attended an initial laboratory session that started at either 1000h (69.4%) or 1400h. During the initial laboratory visit, children participated with a female experimenter in 12 standardized tasks selected from the Laboratory Temperament Assessment Battery (Lab-TAB; Goldsmith, Reilly, Lemery, Longley, & Prescott, 1995), which includes tasks designed to elicit a range of behavioral and emotional expressions from the child. Parents were asked to refrain from feeding their child for one hour prior to coming to the laboratory, and from giving their child caffeinated products for two hours prior, and dairy products 15 minutes prior, to the session, as these factors are known to alter cortisol values (Gunnar & Talge, 2008).

The timing of the salivary cortisol samples was determined based on findings that salivary cortisol levels reflect the level of stress experienced in the prior 20–40 minutes (Dickerson & Kemeny, 2004; Gunnar & Talge, 2008) and on previous studies using similar stress-inducing paradigms, which have been shown to be sensitive to individual differences in cortisol reactivity in preschool-age children (Luby et al., 2003; Talge, Donzella, & Gunner, 2008). In order to obtain a baseline sample, the first sample was collected 20 minutes following adaptation to the laboratory during which time the child played quietly with the experimenter. This period of adaptation was based on previous developmental neuroendocrine research in young children (Gunnar & Talge, 2008; Talge et al., 2008). The second sample was collected 30 minutes following the Stranger Approach task of the Lab-TAB, during which the child was separated from his/her parent and a stranger entered the room. The third salivary cortisol sample was taken 60 minutes after the Stranger Approach task, which was 30 minutes after a qualitatively different frustration-inducing laboratory stressor (i.e., the child not being able to unlock a transparent box with a desirable toy inside). The final sample was collected 20 minutes after the final Lab-TAB task, which was another frustration-inducing laboratory stressor. During this task, the child was left alone with a wrapped empty box to open, under the pretense that an appealing toy was inside. After two minutes, the experimenter returned and explained that she forgot to put the child's gifts in the box and presented the gifts to the child. Lastly, during a scheduled break in between tasks, the child's buccal cells were collected for genetic analysis.

[1]Findings were similar when non-Caucasian children or children of unknown ethnicity were included in all analyses.

Measures

Salivary cortisol—Saliva for cortisol determination was obtained by having children dip 2 in. long cotton dental rolls into .025g of cherry Kool-Aid® mix. Children then placed the cotton in their mouths until saturated. These procedures are known to have little-to-no effect on cortisol concentrations given the assay procedures used (Talge, Donzella, Kryzer, Gierens, & Gunnar, 2005). The wet cotton was collected and then expressed into vials for storage at −20 C until assayed. Samples were later shipped to the Biochemistry Laboratory at the University of Trier, Germany. Samples were assayed in duplicate, using a time-resolved fluorescence immunoassay with flourometric end point detection (DELFIA). Inter- and intra-assay coefficients of variation are 7.1% – 9.0% and 4.0% – 6.7%, respectively.

As indicated above, four samples were taken in the laboratory and the timing of the cortisol samples was based on the presumed stress-inducing qualities of the tasks. The initial sample was taken after consent and acclimation to the laboratory (0 min), 30 minutes after the Stranger Approach task (+60 min after the initial sample), 60 minutes after Stranger Approach (+90 min), and 20 minutes after the final Lab-TAB task (+130 min).

Genotyping—Children's buccal cells were collected for genetic analysis by rubbing the inside of each participant's cheek with two swabs (Epicentre Biotechnologies MasterAmp™ Buccal Swab Kits, Madison, WI). Genomic DNA was successfully extracted for all 156 children. For the purposes of another study, two laboratories independently genotyped 92.3% of the sample. There was 100% agreement for the *5-HTTLPR* polymorphism and 1 discrepant genotype for *BDNF* Val66Met. This subject was excluded, as were 11 children of non-White or unknown ethnicity, leaving a sample of 144 children for the present study.

The genotypes for *5-HTTLPR* and *BDNF* were obtained following previously published protocols (Hunnerkopf, Strobel, Gutknecht, Brocket, & Lesch, 2007; Lesch et al., 1996). Briefly, for *5-HTTLPR*, the polymorphic region was amplified by polymerase chain reaction (PCR) with oligonucleotide primers: forward-5'-GAG GGA CTG AGC TGG ACA AC-3'; reverse-5'-GCA GCA GAC AAC TGT GTT CAT C-3'. PCR started with an initial denaturation at 95° C for 3 minutes, followed by 45 seconds at 95° C, 45 seconds at 61.2° C, 45 seconds at 72° C for 30 cycles, and a final extension at 72° C for 3 minutes. PCR products were separated on a 3% agarose gel containing ethidium bromide, and bands were visualized under UV light. For the *5-HTTLPR* genotype, the resulting product with 585 bp carries the short allele (*s*), whereas the resulting product with the 629 bp carries the long allele (*l*). Of the 144 children, 28 (19.4%) were homozygous for the short allele (*s*/*s*), 71 (49.3%) were heterozygous (*s*/*l*), and 45 (31.2%) were homozygous for the long allele (*l*/*l*). The distribution of genotypes is in Hardy-Weinberg equilibrium. Our primary *5-HTTLPR* genotype analyses compared children who were homozygous for the short allele to those with at least one copy of the long allele (*s*/*s* vs. *s*/*l* or *l*/*l*), given previous findings that youth homozygous for the *s* allele exhibited heightened cortisol reactivity (Gotlib et al., 2008; Jabbi et al., 2007). However, following Lesch et al. (1996), we also compared participants with at least one short allele to those who were homozygous for the long allele on HPA axis reactivity (*s*/*s* or *s*/*l* vs. *l*/*l*).

For *BDNF*, the polymorphic region was first amplified by PCR with oligonucleotide primers: forward-5'-AAA GAA GCA AAC ATC CGA GGA CAA-3'; reverse-5'-ATT CCT CCA GCA GAA AGA GAA GAG G-3'. PCR started with an initial denaturation at 95° C for 3 minutes, followed by 45 seconds at 95° C, 45 seconds at 62° C, 45 seconds at 72° C for 35 cycles, and a final extension at 72° C for 3 minutes. The resulting 274 bp fragment was incubated for three hours with the restriction enzyme *NlaIII* (New England Biolabs, Ipswich, MA). The resulting digested PCR products were separated on a 5% agarose gel containing ethidium bromide, and bands were visualized under UV light and designated as *Met* or *Val*. In our sample, 94 children (65.3%) were homozygous for the *Val/Val* genotype, 41 (28.5%) were heterozygous, and 9

(6.2%) were homozygous for the *Met/Met* genotype, which is consistent with reports of the allelic distribution in White samples (Shimizu et al., 2004); the distribution of genotypes is in Hardy-Weinberg equilibrium. Analyses contrasted children with at least one *Met* allele with those with the *Val/Val* genotype, as the *Met* variant is associated with alterations in brain anatomy, memory, and reduced neuronal BDNF secretory activity than the *Val/Val* genotype (Bath & Lee, 2006; Chen et al., 2006).

Potential confounds—Several factors were assessed as potential confounds on children's cortisol levels: age, gender, time of laboratory visit, hours of sleep the night prior to the laboratory sampling, observer-rated activity level during the laboratory visit, and parent-reported internalizing (α = .84) and externalizing problems (α = .90) as assessed using the Child Behavior Checklist/1½–5 (CBCL/1½ –5; Achenbach & Rescorla, 2000). Child activity level (the vigor, energy, and the extent of the child's physical movements) was coded on a single scale for each episode of the Lab-TAB and averaged across episodes to examine the influence of activity level on children's cortisol levels. Both the internal consistency across episodes (α = .73) and interrater reliability (intraclass correlation coefficient [ICC] = .85, assessed on a subsample of 28 cases) were acceptable.

Data analysis strategy

Cortisol data were log10 transformed prior to analysis to correct for positive skew (Gunnar & Talge, 2008) and treated as the dependent variable. Three cortisol outliers (>44 nmol/L) were removed from the data: one from the initial sample and two from the final sample.

Estimates of children's cortisol reactivity based on each child's four cortisol samples over the course of the laboratory session were obtained using multilevel growth curve modeling. A two-level multilevel analysis (Singer & Willet, 2003) was performed to model children's cortisol activity in response to the laboratory stressors and to examine associations between genetic polymorphisms and the components of the growth curve defining individual differences in cortisol reactivity (i.e., intercept, linear slope, and quadratic curvature). Multilevel or mixed effects modeling was selected as it accounts for the nested data structure (cortisol levels nested within individuals) and adjusts for correlated error within each level. The multilevel model was estimated using the Hierarchical Linear Modeling (HLM) statistical program version 6 (SSI Inc., Lincolnwood, IL).

Two levels of analysis were estimated. Level 1 estimates individual-level change in cortisol over time. Specifically, we estimated each child's cortisol levels at arrival to the laboratory (i.e., intercepts) and his/her change in cortisol in response to the laboratory stressors (i.e., linear slope and quadratic curvature). Both linear and quadratic growth models were used to examine a within-subjects regression of an individual's cortisol reactivity onto the time of each assessment. Level 2 examines person-level differences in change. On the second level, we examined person-level genetic predictors and their interaction on cortisol activity, along with several covariates that may influence cortisol levels. The person-level covariates included: time of visit, hours of sleep the night prior to the assessment, activity level, and internalizing and externalizing problems. Any significant covariates were included in the final model to test whether they account for the relation between genetic polymorphisms and cortisol activity.

Three adjustments were made to the data to ease interpretation of the results. First, time was anchored at the first cortisol sample (time = 0) so that the cortisol intercepts would reflect the average individual's cortisol level at arrival to the laboratory. Second, all Level 2 between-person variables were centered at their grand mean. Third, we used a pairwise missing data procedure to handle any missing data at Level 1 so the sample size varied slightly by analysis. *N*'s are reported for each analysis.

Results

Descriptive analyses

Table 1 shows the means, standard deviations, and *N*'s for the covariates and cortisol levels in nanomoles per liter (nmol/L) for the total sample and by *5-HTTLPR×BDNF* genotypes. We examined whether the *5-HTTLPR (ss, sl, ll)*, *BDNF (met/met, met/val, val/val)*, and *5-HTTLPR×BDNF* genotypes differed on any confounding variable listed in Table 1. No significant differences between genotypes were observed.

Multilevel analyses examining differences in cortisol response activity

As seen in Table 2, the baseline trajectory model demonstrated that there was a significant linear decrease in cortisol values from arrival to the laboratory to children's cortisol levels following the separation stressor (sample 2). Following the separation stressor, children's cortisol levels began to rise for sample 3 (30 minutes after a frustrating task) and sample 4 (20 minutes after the last Lab-TAB task), as indicated by a significant positive quadratic effect (i.e., concave up). The growth curve measuring children's cortisol reactivity and the salivary cortisol values, obtained in this investigation, were similar to other studies of preschoolers using analogous stress-inducing laboratory tasks conducted at similar times of the day (Luby et al., 2003;Talge et al., 2008), and similar decreases in response to stressors have been observed in other studies (e.g., Gotlib et al., 2008;Gunnar, Frenn, Wewerka, & Van Ryzin, 2009; for a review, see Gunnar, Talge, & Herrera, 2009).

As seen in Table 2, the random error terms associated with the intercept, linear, and quadratic components were significant, demonstrating variability among children's cortisol activity across the visit, which supports the examination of between-person predictors of each of these components.

Potential confounds in children's cortisol activity

Prior to examining the genetic predictors of the growth curve, we examined potential confounds. As seen in Table 2, time of laboratory visit, age, and hours of sleep were significantly associated with components of the growth curve, whereas gender, children's internalizing and externalizing symptoms and activity level were not significantly associated with the growth curve. The significant association between time of visit (1000h vs. 1400h) and the intercept of the growth curve reflects the diurnal rhythm in cortisol levels across the day (i.e., higher levels after awakening and lower levels later in the day).

Genetic polymorphisms and cortisol reactivity

For each analysis, we included the following Level 2 covariates: time of visit, age, gender, and hours of sleep. We did not observe any significant interactions with gender. Results were similar whether hours of sleep were included as a covariate or not; therefore, we removed it from analyses as it limited the sample size as a result of missing data for that variable. We examined the main effects of *5-HTTLPR* and *BDNF* on children's cortisol reactivity. Along with time of visit, age, and gender, both *5-HTTLPR* and *BDNF* were included at each Level 2 parameter (i.e., intercept, linear slope, quadratic curvature). *BDNF* was not significantly associated with any component of the growth curve, and *5-HTTLPR s/s* alleles were significantly associated with a greater positive increase in curvature.

The main effect for *5-HTTLPR* was qualified by the interaction between *5-HTTLPR* and *BDNF*. As seen in Table 3, the interaction between *5-HTTLPR* and *BDNF* was significantly associated with the intercept, slope and curvature of the growth curve. In order to probe the interaction, we created four Level 2 dummy variables to capture the following genotypes: (1) *5-HTTLPR s/s* and *BDNF Val/Met* or *Met/Met* (N=9); (2) *5-HTTLPR s/s* and *BDNF Val/Val*

(N=19); (3) *5-HTTLPR s/l* or *l/l* and *BDNF Val/Met* or *Met/Met* (N=41); (4) *5-HTTLPR s/l* or *l/l* and *BDNF Val/Val* (N=75).

As seen in Figure 1, children homozygous for the *short-5-HTTLPR* allele and carrying the *Met-BDNF* allele had significantly lower cortisol levels at arrival compared to children homozygous for both the *short-5-HTTLPR* and the *Val-BDNF* alleles (β = −.169, *SE* = .081, *t(25)* = −2.089, *p* = .047); children carrying the *long-5-HTTLPR* and the *Met-BDNF* alleles (β = −.261, *SE* = .078, *t(47)* = −3.331, *p* = .002); and children carrying the *long-5-HTTLPR* and who were homozygous for the *Val-BDNF* alleles (β = −.181, *SE* = .061, *t(81)* = −2.990, *p* = .004);. In addition, children homozygous for the *short-5-HTTLPR* allele and carrying the *Met-BDNF* allele demonstrated continuously increasing cortisol levels across the lab visit as indicated by significant associations with the slope (β = .238, *SE* = .092, *t(25)* = 2.586, *p* = .016) and curvature (β = −.053, *SE* = .019, *t(103)* = −2.696, *p* = .009) compared to children homozygous for both the *short-5-HTTLPR* and the *Val-BDNF* alleles.

In contrast, children homozygous for both the *short-5-HTTLPR* and the *Val-BDNF* alleles had greater initially declining cortisol levels followed by an attenuated rise in cortisol compared to children who were homozygous for the *short-5-HTTLPR* allele and carrying the *Met-BDNF* allele; children carrying the *long-5-HTTLPR* allele and the *Met-BDNF* allele; and children carrying the *long-5-HTTLPR* allele and who were homozygous for the *Val-BDNF* allele. Specifically, children homozygous for the *short-5-HTTLPR* and the *Val-BDNF* alleles had a greater decline in cortisol levels compared to children carrying the *long-5-HTTLPR* allele and the *Met-BDNF* allele, as evidenced by significant associations with the slope (β = −.102, *SE* = .085, *t(57)* = −2.106, *p* = .039) and curvature (β = .032, *SE* = .010, *t(57)* = 3.115, *p* = .003) of the growth curve. Likewise, children homozygous for the *short-5-HTTLPR* and the *Val-BDNF* alleles also had a greater decline in cortisol levels compared to children carrying the *long-5-HTTLPR* allele and who were homozygous for the *Val-BDNF* allele, as evidenced by significant associations with the slope (β = −.125, *SE* = .043, *t(91)* = −2.928, *p* = .005) and curvature (β = .035, *SE* = .010, *t(91)* = 3.943, *p* = .000) of the growth curve.

No other genotypes significantly differed from one another. The *5-HTTLPR-BDNF* interaction was not observed when the *s/s* and *s/l* groups were combined and compared to the *l/l* group. Lastly, given that the time of the laboratory session was a significant confound, we repeated our analyses with participants who attended the 1000h laboratory session only (~ 70% of sample), and results were similar.

Discussion

This study is the first to demonstrate the epistatic effects of *5-HTTLPR* and *BDNF* on HPA axis activity. In a community sample of preschoolers, we found that the homozygous *short-5-HTTLPR* genotype was associated with different patterns of stress reactivity as a function of *BDNF*. Children homozygous for the *short-5-HTTLPR* and carrying the *Met-BDNF* allele evidenced a lower initial cortisol level followed by a positive increase in cortisol from the laboratory stressors. In contrast, children who were homozygous for both the *short-5-HTTLPR* allele and the *Val-BDNF* allele exhibited a greater decline in cortisol in response to the laboratory stressor. Children carrying the *long-5-HTTLPR* genotype did not significantly differ from one another as a function of *BDNF* and exhibited flat growth curves, with non-significant effects. Our findings demonstrated that genetic polymorphisms influenced individual differences in reactivity and/or regulation of the HPA axis in response to a laboratory stressor.

In order to understand the differential cortisol response patterns we observed, we need to address issues that arise when assessing cortisol reactivity in young children (Gunnar & Talge,

2008; Gunnar, Talge, et al., 2009). In developmental neuroendocrine research, studies have typically failed to show a mean positive increase in cortisol responses to laboratory stressors from late infancy throughout middle childhood (Gunnar, Talge, et al., 2009). It has been hypothesized that this may reflect a stress-hyporesponsive period in the development of the HPA system. Alternatively, it may be a function of the inadequacy of laboratory stress-inducing paradigms employed with young children, which are confined by ethical and developmental constraints (Gunnar, Talge, et al., 2009). Additionally, obtaining pre-stress baseline cortisol samples in developmental research is particularly difficult as the time required to achieve a valid baseline in young children remains unclear and young children typically cannot tolerate long laboratory sessions that incorporate extended adaptation periods (Gunnar & Talge, 2008). We chose to incorporate a developmentally appropriate adaptation period (Talge et al., 2008); however, it is possible that it was not sufficient and may reflect cortisol responses to coming to the lab or other unknown factors present prior to the laboratory visit; therefore, consistent with recent developmental neuroendocrine research, we interpret the initial sample, along with the subsequent cortisol samples, as part of the continuous reactivity of the HPA axis (Gunnar & Talge, 2008).

In spite of these methodological issues present in developmental neuroendocrine studies, researchers have sought to delineate individual differences in children's cortisol reactivity in order to identify individuals who may be more vulnerable to heightened or prolonged HPA activation (Gunnar & Quevedo, 2007; Gunnar & Vazquez, 2006). Specifically, a large body of research has established the influence of social-environmental factors (parenting, peers, maltreatment) and within-person factors (i.e., emotions and behaviors) on individual differences in children's HPA axis reactivity (Gunnar & Donzella, 2002; Gunnar & Quevedo, 2007). However, none of the prior research has investigated the role of genetic polymorphisms on children's HPA axis reactivity, which is particularly important given the significant heritability of the HPA axis (Bartels et al., 2003; Frederenko et al., 2004), and as both genetic and environmental factors likely play a role in HPA axis reactivity (e.g., Alexander et al., 2009).

The study provided some support for our hypothesis that children homozygous for the *short-5-HTTLPR* and carrying the *Met-BDNF* allele would demonstrate greater cortisol reactivity than other children. This genotype group exhibited a positive cortisol increase in response to the laboratory stressors, whereas the other genotype groups evidenced decline in cortisol or were unresponsive to the laboratory stressors. These findings are consistent with the hypothesis that the HPA axis is a mechanism through which these genes influence stress sensitivity at the behavioral level (Kaufman et al., 2006; Kim et al., 2007). Interestingly, this same subgroup of children demonstrated a similar pattern of cortisol responses found in depressed preschoolers (Luby et al., 2003), which suggests that these responses coincide with increasing risk. These genes were also related to lower initial cortisol levels during the pre-stress sampling. While this finding is contrary to our hypothesis, a similar finding has been reported in animals. Peer-raised *5-HTTLPR* heterozygous macaques evidenced blunted pre-stress HPA-axis activity, as well as increased stress reactivity (Barr et al., 2004). On the one hand, this may be indicative of abnormalities of the HPA system (Gunnar & Vazquez, 2001). On the other hand, this differential responsivity may indicate that these genes have greater plasticity or susceptibility to context. In other words, the *ss-5-HTTLPR/Met-BDNF* genotype is less reactive under low stress conditions and more reactive under high stress conditions (Belsky et al., 2009; Ellis & Boyce, 2008).

We also found that children homozygous for the *short-5-HTTLPR* and the *Val-BDNF* allele evidenced greater decreases in cortisol in response to the stressors. When our findings are put in the context of gene X environment interactions involving *5-HTTLPR* and *BDNF* (Kaufman et al., 2006; Kim et al., 2007), it appears that this subgroup may be more resilient to

environmental adversity. Likewise, a recent study by Gunnar, Frenn, and colleagues (2009) found that children who were able to maintain normal growth patterns despite exposure to early life stress evidenced reduced cortisol responsiveness to a laboratory stressor, which they interpret as a reflection of resiliency. Lastly, children carrying a *long5-HTTLPR* allele, regardless of *BDNF* genotype, were unresponsive to the laboratory stressors, which complements findings that the *long* allele is associated with less brain activation to environmental threat (Munafo et al., 2008).

In this study, the relation of *5-HTTLPR* and *BDNF* genotypes with HPA axis activity in preschoolers did not differ by gender, as has been previously reported in some adult populations (Jabbi et al., 2007; Shalev et al., 2009; Wust et al., 2009). Nevertheless, our findings are consistent with research demonstrating that gender differences in HPA axis function typically do not emerge until later in development, usually coinciding with puberty (Gunnar & Vazquez, 2006; Gunnar, Wewerka, Frenn, Long, & Griggs, 2009; Kirschbaum, Wust, & Hellhammer, 1992; Knutsson et al., 1997; Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004). Therefore, it is possible that the influence of genes on HPA axis function may vary across development, particularly around puberty, and differ by gender.

This study had several limitations. First, our sample was small, which is problematic as our findings were based on groups stratified by gene-gene interactions. We cannot rule out the possibility that the results are due to chance; therefore, our findings require replication. The limited sample size also precluded any examination of three-way interactions with gender or environmental factors. Third, we did not analyze recently reported subtypes of the *5-HTTLPR* long alleles that may have functional significance for stress reactivity (Wendland, Martin, Kruse, Lesch, & Murphy, 2006). Fourth, our sample was ethnically homogenous. We cannot generalize results to economically disadvantaged groups or other racial/ethnic groups. Fifth, salivary cortisol response to stress was our only measure of HPA axis regulation. Future research should assess multiple aspects of HPA axis function, including basal cortisol secretion (e.g., the cortisol awakening response), adrenocorticotropic hormone (ACTH) levels, and dexamethasone nonsuppression. Lastly, the study was cross-sectional. Long-term follow-up is necessary to test the hypothesis that these factors contribute to stress sensitivity or resiliency at the behavioral level and in the development of stress-related disorders.

In sum, a large body of research has demonstrated that stress and the HPA axis play a critical role in numerous psychiatric and medical diseases (McEwen, 2008); therefore, understanding factors that influence HPA axis function, specifically stress sensitivity, is of critical importance. This study is the first to demonstrate the epistatic effects of *5-HTTLPR* and *BDNF* on HPA axis reactivity to stress and is also the first to investigate the genetics of HPA axis functioning in very young children. The presence of these associations during early childhood is particularly noteworthy, as exposure to adversity during early development appears to contribute to lasting neurobiological changes that increase risk for psychopathology in adulthood (Brown & Harris, 2008; Heim, Newport, Mletzko, Miller, & Nemeroff, 2008). At the same time, other early environmental factors, such as nurturing care-giving environments, may protect developing neurobiological systems, even in those at high genetic risk (Kaufman et al., 2006). Future research should work to identify both genetic and environmental factors contributing to the development of stress regulation across the lifespan, which may help delineate how stress increases disease susceptibility in certain individuals.

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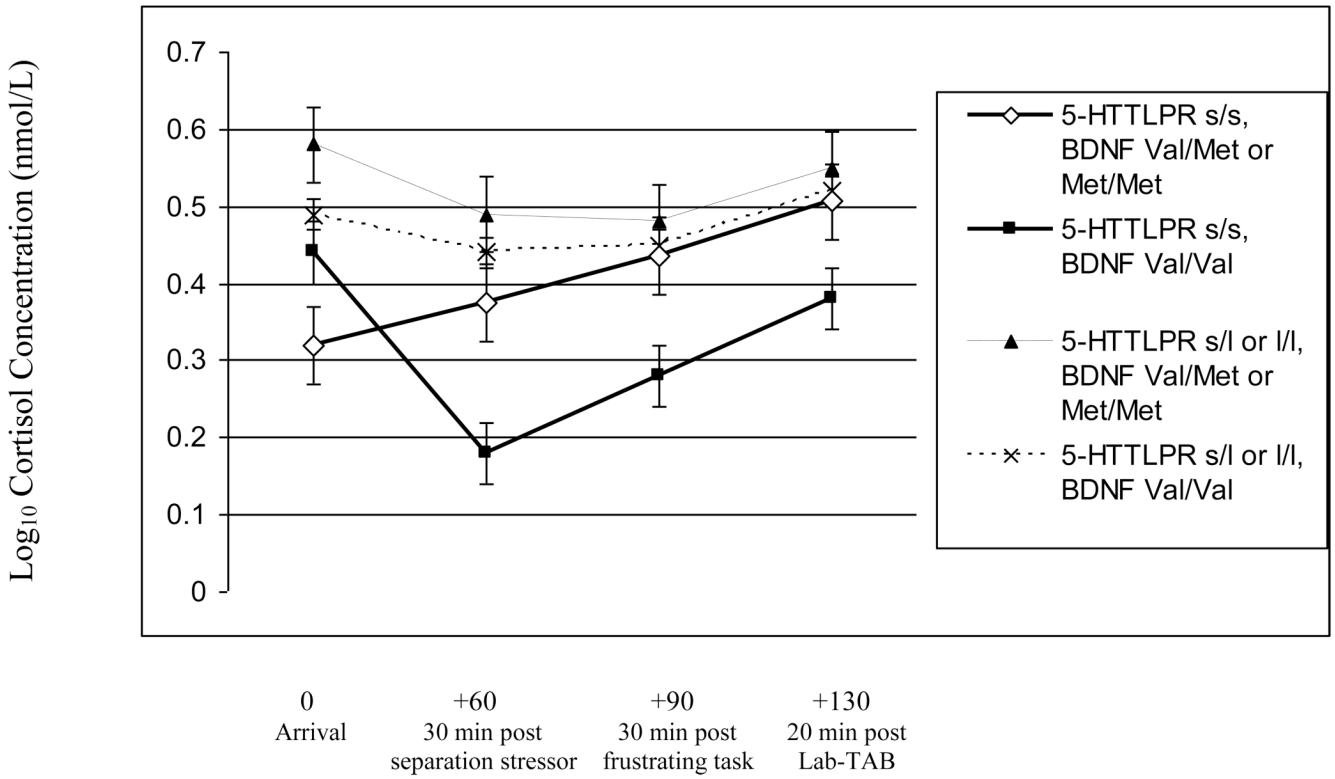

Figure 1.

Multilevel regression coefficients indicating children's $\log_{10}$ transformed cortisol levels in nanomoles/Liter (nmol/L) as a function of serotonin transporter gene polymorphism (*5-HTTLPR*) and brain-derived neurotrophic factor (*BDNF*) genotypes (*s* indicates short allele and *l* indicates long allele; *Val* indicates valine and *Met* indicates methionine). Regression coefficients were adjusted to account for time of cortisol data collection. Bars reflect standard error of measurement.

Table 1

Descriptive statistics of sample

Variable	Total sample	*5-HTTLPR s/s* and *BDNF Val/Met* or *Met/Met*	*5-HTTLPR s/s* and *BDNF Val/Val*	*5-HTTLPR s/l* or *l/l* and *BDNF Val/Met* or *Met/Met*	*5-HTTLPR s/l* or *l/l* and *BDNF Val/Val*
N	144	9	19	41	75
Gender (male/female)	72/72	8/1	8/11	18/23	38/37
Age (months)	43.50 (2.79)	43.44 (3.91)	44.63 (3.30)	43.37 (2.71)	43.29 (2.54)
1000h visit/ 1400h visit	100/44	6/3	15/4	29/12	50/25
CBCL internalizing problems	9.01 (6.42)	8.00 (4.17)	8.75 (6.88)	9.09 (5.20)	9.27 (7.21)
CBCL externalizing problems	12.66 (7.22)	15.09 (9.45)	11.94 (6.80)	11.37 (6.19)	13.32 (7.67)
Global activity score	1.58 (.31)	1.62 (.24)	1.48 (.34)	1.57 (.34)	1.61 (.30)
Hours of sleep	10.28 (1.44)	10.56 (.85)	9.97 (1.12)	10.42 (1.32)	10.24 (1.64)
Cortisol level at time 1 (nmol/L)	4.134 (5.829)	2.048 (.852)	3.687 (4.605)	5.487 (7.954)	3.794 (4.993)
	N = 143	N = 9	N = 19	N = 40	N = 75
Cortisol level at time 2 (nmol/L)	3.840 (5.703)	2.752 (1.978)	2.114 (.8040)	5.532 (8.471)	3.485 (4.566)
	N = 144	N = 9	N = 19	N = 41	N = 75
Cortisol level at time 3 (nmol/L)	3.807 (3.959)	3.369 (1.179)	3.583 (4.364)	4.776 (5.940)	3.364 (2.387)
	N = 144	N = 9	N = 19	N = 41	N = 75
Cortisol level at time 4 (nmol/L)	5.193 (3.423)	4.348 (2.121)	5.785 (2.118)	5.920 (4.715)	4.768 (2.949)
	N = 142	N = 9	N = 19	N = 39	N = 75

Notes. *5-HTTLPR* = serotonin transporter promotor region polymorphism: *s* = short and *l* = long. *BDNF* = brain-derived neurotrophic factor Val66Met polymorphism: Val = valine and Met = methionine. CBCL = Parent-reported Child Behavior Checklist 1½ –5; nmol/L = nanomoles/Liter.

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Table 2

Univariate multilevel analyses of associations between potential confounds and cortisol activity

Fixed effect	**β**	***SE***	***T***	***P***	**N**
Cortisol intercept	.466	.025	18.400	.000	144
Time of visit	−.104	.051	−2.069	.040	144
Age	−.015	.008	−1.963	.051	144
Gender	−.060	.050	−1.195	.235	144
Hours of sleep	−.016	.015	−1.043	.299	133
Global activity level	.016	.066	.240	.811	144
CBCL internalizing problems	.001	.003	.321	.749	136
CBCL externalizing problems	.000	.003	.014	.988	136
Cortisol linear slope	−.087	.017	−5.248	.000	144
Time of visit	.057	.036	1.569	.119	144
Age	.004	.006	.851	.397	144
Gender	.025	.033	.770	.443	144
Hours of sleep	.022	.008	2.594	.011	133
Global activity level	.018	.050	.347	.729	144
CBCL internalizing problems	−.003	.003	−1.119	.266	136
CBCL externalizing problems	−.000	.003	−.092	.927	136
Cortisol quadratic curvature	.030	.004	8.006	.000	144
Time of visit	−.015	.008	−1.913	.057	144
Age	−.000	.001	−.301	.764	144
Gender	−.008	.008	−1.005	.317	144
Hours of sleep	−.005	.002	−2.693	.008	133
Global activity level	−.004	.022	−.438	.662	144
CBCL internalizing problems	.001	.001	1.439	.152	136
CBCL externalizing problems	.000	.001	.266	.791	136
Random effect of unconditional growth curve	Variance Component	*SD*	χ^2	*P*	*df*
Level 1 intercept	.081	.284	1094.736	.000	143
Linear slope	.026	.163	432.862	.000	143

Fixed effect	β	*SE*	*T*	*P*	N
Quadratic curvature	.001	.037	427.915	.000	143

Notes. All fixed effects are robust standard errors. All Level 1 predictors are uncentered. Level 2 variables are grand mean centered. *SE* = standard error; *SD* = standard deviation; Time of visit: 1000h = 0 and 1400h = 1; Gender: Males = 0 and Females = 1; CBCL = parent-report Child Behavior Checklist 1½–5.

Table 3

Multilevel model of associations between 5-HTTLPR and BDNF genotypes and children's cortisol activity

Fixed effect	**β**	***SE***	***T***	***P***
Cortisol intercept	.466	.024	19.297	.000
Time of visit	−.081	.050	−1.622	.125
Age	−.014	.008	−1.638	.103
Gender	−.078	.051	−1.541	.125
5-HTTLPR	.004	.071	.063	.951
BDNF	.087	.066	1.326	.187
5-HTTLPR×BDNF	−.301	.113	−2.656	.009
Cortisol linear slope	−.087	.016	−5.580	.000
Time of visit	.038	.035	1.085	.280
Age	.007	.005	1.365	.175
Gender	.040	.033	1.210	.229
5-HTTLPR	−.140	.043	−3.259	.002
BDNF	−.025	.038	−.642	.521
5-HTTLPR×BDNF	.272	.102	2.670	.009
Cortisol quadratic curvature	.030	.004	8.630	.000
Time of visit	−.010	.007	−1.416	.159
Age	−.001	.001	−.838	.404
Gender	−.010	.007	−1.332	.185
5-HTTLPR	.038	.009	4.226	.000
BDNF	.006	.009	.649	.517
5-HTTLPR×BDNF	−.060	.023	−2.641	.010
Random effect of unconditional growth curve	Variance Component	*SD*	χ^2	*P*
Level 1 intercept	.076	.275	985.294	.000
Linear slope	.023	.152	377.517	.000
Quadratic curvature	.001	.034	360.028	.000

Note. N = 144; for *t* tests, *df* = 137. All fixed effects are with robust standard errors. All Level 1 predictors are uncentered; Level 2 variables are grand mean centered. *SE* = standard error; *SD* = standard deviation; Time of visit: 1000h = 0 and 1400h = 1; Gender: Males = 0 and Females = 1; *5-HTTLPR* = serotonin transporter promotor region polymorphism: *s/l* and *l/l* = 0 and *s/s* = 1; *BDNF* = brain-derived neurotrophic factor Val66Met polymorphism: *Val/Val* = 0 and *Val/Met* and *Met/Met* = 1.