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BDNF Val66Met polymorphism is associated with HPA axis reactivity to psychological stress characterized by genotype and gender interactions

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Summary
Background: A key protein in maintaining neuronal integrity throughout the life span is brain-derived neurotrophic factor (BDNF). The *BDNF* gene is characterized by a functional polymorphism, which has been associated with stress-related disorders such as anxiety-related syndromes and depression, prompting us to examine individual responses by Genotype and Sex to a standardized social stress paradigm. Gender differences in BDNF × stress responses were posited because estrogen induces synthesis of BDNF in several brain regions.
Methods: 97 university students (51 females and 46 males) participated in a social stress procedure (Trier Social Stress Test, TSST). Indices of stress were derived from repeated measurement of cortisol, blood pressure, and heart rate during the TSST. All subjects were genotyped for the Val66Met polymorphism.
Results: Tests of within-subject effects showed a significant three-way interaction (SPSS GLM repeated measures: Time (eight levels) × BDNF (val/val, val/met) × Sex: $p = 0.0002$), which reflects gender differences in the pattern of cortisol rise and decline during the social challenge. In male subjects, val/val homozygotes showed a greater rise in salivary cortisol than val/met heterozygotes. In female subjects, there was a trend for the opposite response, which is significant when area under the curve increase (AUCi) was calculated for the val/val homozygotes to show the lowest rise. Overall, the same pattern of results was observed for blood pressure and heart rate.

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Conclusions: These results indicate that a common, functionally significant polymorphism in the *BDNF* gene modulates HPA axis reactivity and regulation during the TSST differently in men and women. Findings may be related to gender differences in reactivity and vulnerability to social stress.

1. Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is a major pathway for regulating stress responses, and animal and human studies have shown that exaggerated response as well as under-response of the HPA axis are associated with a spectrum of disease conditions (McEwen, 2008). A host of publications have shown that brain and body adaptations to acute and chronic stress are critical for physical and mental health (McEwen, 2008).

There is considerable impact of heredity on basal free cortisol, and analyses of results across five comparable twin studies estimated heritability to be 62% for this measure (Bartels et al., 2003). Similarly, HPA axis responses to moderate psychosocial stress elicited during the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) have been shown to be moderately heritable (Federenko et al., 2004). The TSST protocol is probably the most widely used protocol for elicitation of social stress in adults. The protocol is comprised of role-playing and a cognitive task performed in front of stone-faced "interviewers" and reliably evokes endocrine responses in adult samples. The task elicits substantial HPA responses because it combines both uncontrollable and social-evaluative elements (Dickerson and Kemeny, 2004). Further, the frequent use of the protocol in research studies affords comparison of results across studies.

Previous studies have shown that stress responses elicited during the TSST procedure are associated with gene variants, including the glucocorticoid receptor (*GR*) gene (Wust et al., 2004), the *GABRA6* gene (Uhart et al., 2004) and the Mu-Opioid receptor gene (Chong et al., 2006), and gender differences have been shown on some TSST outcome measures. However, to our knowledge, only one study has examined gene effects on stress responses elicited in the TSST paradigm as a function of gender (Kumsta et al., 2007), despite the fact that significant gender differences have been reported in relation to overt behaviors shown during the TSST and other stress paradigms (Kirschbaum et al., 1993).

A gene of considerable interest, which we hypothesized may regulate the HPA axis response to social stress, is the brain-derived neurotrophic factor (BDNF). As a member of the neurotrophin family (Dechant and Neumann, 2002) (together with nerve growth factor (NGF) and neurotrophin-3 to -5), it influences almost all aspects of development in the CNS during early life and is involved later in life in the survival, differentiation, and plasticity of the CNS (Ridder et al., 2005; Lipsky and Marini, 2007). Importantly, early stress can alter the threshold for HPA reactivity in humans and nonhuman animals, and therefore genes related to physiological plasticity are of considerable interest. Also relevant are the findings suggesting that BDNF protects against stress-induced neuronal damage (Bergstrom et al., 2008).

The *BDNF* gene, like other peptide growth factors, encodes a precursor peptide (proBDNF), which is proteolytically cleaved to form the mature protein (Seidah et al., 1996). Only one frequent, non-conservative polymorphism in the human *BDNF* gene (dbSNP number rs6265) has been identified, a single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) producing an amino acid substitution (valine to methionine) at codon 66 (Val66Met). Several investigations using brain imagery and transfection experiments have demonstrated the functional importance of the Val66Met polymorphism on BDNF expression levels (Egan et al., 2003; Bueller et al., 2006). Furthermore, the Val66Met polymorphism has been associated with stress-related dysfunction, such as anxiety-related personality traits (Lang et al., 2005) and depression, both in animal models (Monteggia et al., 2007) as well as in humans (Gotlib et al., 2007).

The role of BDNF in maintaining neuronal integrity across the life span, its relation to neuronal integrity and plasticity, its suggested role in the etiology of some stress-related traits and disorders (Gotlib et al., 2007; Groves, 2007), as well as the presence of a common *BDNF* gene variant that modulates expression, suggested to us that the Val66Met polymorphism would be worth investigating for its role in modulating salivary cortisol and autonomic responses in a group of non-clinical subjects participating in the TSST paradigm. Additionally, the sex bias observed in clinical depression (Grigoriadis and Robinson, 2007), the sex differences in depression-related behaviors in BDNF conditional knockout mice (Monteggia et al., 2007), the effects of estrogen on brain BDNF (Sasahara et al., 2007; Meltser et al., 2008), and the higher reactivity of the HPA axis to social stress in men compared to women (Kirschbaum et al., 1993) prompted us to hypothesize that the BDNF Val66Met variant would show a differential effect on acute social stress responses in men and women.

2. Materials and methods

2.1. Subjects

Participants were primarily college students at Israeli institutions of tertiary education, recruited by word of mouth and advertisements on campus notice boards for a study on human genetics and personality. Selection criteria stipulated that subjects were <35 years old, had no history of psychiatric or endocrine illness (confirmed by a screening interview but not a structured interview), were currently non-smokers, were not pregnant, had not given birth in the past year, and were not using medication on a regular basis besides single-phase oral contraceptives. All together, 102 subjects participated in the experiment. The data of five subjects were not included in the final analyses because two of them had rare alleles (met/met) and the other three did not provide sufficient saliva for reliable measurement. The final sample ($N = 97$) was comprised of 46 males and 51 females (mean age = 25.29, S.D. = 3.6; mean body mass index (BMI) = 22.53, S.D. = 2.8). Demographics of the sample are presented in

Table 1 Demographics of the different genotype by gender groups.

Genotype	Female ($N = 51$)		Male ($N = 46$)	
	val/val	val/met	val/val	val/met
Sample size	37	14	31	15
Age (S.D.)	25.20 (3.56)	25.61 (5.45)	25.10 (3.25)	25.40 (2.66)
Body mass index (BMI) (S.D.)	22.37 (3.12)	22.85 (3.18)	22.91 (2.71)	21.94 (1.65)

Table 2 Comparison of cortisol measures of women using oral contraceptives (F_OC+, $N = 26$), not using oral contraceptions (F_OC−, $N = 25$), and male subjects ($N = 46$).

	F_OC−	F_OC+	Males	F	p-Value[a]
Mean cortisol across eight time-points (S.E.)	7.16 (0.72)	6.99 (0.70)		0.21	0.646 (ns)
	7.16 (0.72)		10.03 (0.79)	3.88	**0.051**
		6.99 (0.70)	10.03 (0.79)	5.78	**0.019**
AUCi (S.E.)	43.8 (29.1)	55.6 (28.5)		0.01	0.901 (ns)
	43.8 (29.1)		244.3 (45.9)	11.89	**0.001**
		55.6 (28.5)	244.3 (45.9)	11.22	**0.001**

AUCi, area under the curve with respect to increase; S.E., standard error; ns, non-significant.
Values in bold are significant at the 0.05 level.
[a] Values were log-transformed prior to analysis.

Table 1. As shown in Table 2, of the 51 females, 26 were using single-phase oral contraceptive at the time of their participation in the study. The study was approved by the IRB of Herzog Hospital, Jerusalem and all subjects provided written informed consent. Subjects received a modest monetary incentive for participation.

2.2. General procedure

Prior to the testing session, subjects were given two sterile test tubes, each containing 10 ml of Aquafresh mouthwash, for DNA sampling. DNA mouthwashes were returned by mail or hand-delivered to our lab. After receipt of their DNA, subjects were scheduled for testing within a fixed time-window (between 1500 and 1800 h) to counter effects of circadian changes in cortisol. To limit variance, subjects were given explicit instructions to refrain from excessive physical activity for 2 h prior to the experiment and from brushing their teeth, eating, and drinking (besides water) for the 90 min prior to the testing session.

Testing was carried out in a laboratory in the Department of Psychology, Hebrew University. Prior to testing, subjects were asked about their present health and whether they had taken medication (besides oral contraception) during the previous 24 h. Those who described themselves as ill or medicated were rescheduled. The TSST session was carried out as described in the previous studies (Kirschbaum et al., 1993). Briefly, the paradigm entails two parts (5 min each) in which participants role play in front of two stone-faced interviewers and then carry out a challenging counting task.

2.3. Sampling and biochemical analysis

Salivary cortisol was sampled eight times during the 90-min session at the following time-points: 10 min prior to testing, 1 min prior to testing, immediately after testing, and 10, 20, 30, 45, and 60 min post-test. Salivette plugs (Sarstedt, Germany) were used to collect saliva. Saliva samples were kept at room temperature throughout the session and were then immediately centrifuged at 4000 rpm at 24 °C for 10 min and then assayed using the electrochemiluminescence immunoassay with a specific cortisol kit for saliva samples (Roche Diagnostics, USA) in an Elecsys 2010 Analyzer. The lower detection limit of the assay was 0.5 nM/l. Subjects' blood pressure and heart rate were measured using a digital automatic blood pressure monitor (Omron R7).

2.4. DNA extraction and genotyping

DNA was extracted from 20 ml of mouthwash samples using the Master Pure kit (Epicentre, Madison, WI). The SNP (rs6265; A/G; val/met) was genotyped using high-resolution melt (HRM) analysis (see Liew et al., 2004) for a general description of the method). HRM distinguished between val/val, val/met, and met/met amplicons, and the method was verified by comparison of HRM results to those obtained by genotyping a portion of the same samples using the SNaPShot procedure (Applied Biosystems) and analyzing the reaction products on a DNA analyzer (ABI 310). PCR reactions were performed using 5 μl Thermo-Start Master Mix (Thermo scientific), 2 μl primers (2.5 μM), 1 μl SYTO9 (dye), and 1 μl of water to total of 9 μl total volume and an additional 1 μl of genomic DNA. All PCR reactions and HRM analysis were performed on a Rotor-Gene 3000 (Corbett life science, Australia), using the following primers that produced a 142 bp amplicon: F5′GACATCATTGGCTGACACTT′3; R5′CTCCAAAGGCACTTGACTAC′3. PCR reaction conditions were as follows: activating enzyme step at 95.0 °C for 15 min, 45 cycles of denaturation at 95.0 °C for 5 s, reannealing at 58 °C for 15 s and extension at 72 °C for 10 s where at the end of each cycle fluorescence was measured. The reaction proceeded to a hold at 40 °C for 2 min, a second hold at

65 °C for 2 min and then the melt procedure ramped from 65 to 95 °C raising by 0.1 °C every 3 s where fluorescence was acquired. The BDNF genotype frequencies were in Hardy—Weinberg equilibrium.

2.5. Data reduction and final measures

Statistical analyses of cortisol data used raw cortisol values at eight time-points and area under the curve with respect to increase (AUCi), emphasizing the changes over time with respect to basal levels. All values were log-transformed to correct skewed distributions. Area under the curve was calculated by using the trapezoid formula described by Pruessner et al. (2003). Blood pressure and heart rate values were reduced to four measures from 10 min prior to the TSST to 10 min after (samples 1—4) to evaluate the fast sympathetic response (Kirschbaum et al., 1999). Moreover, the systolic and diastolic blood pressures were combined to derive a measure of the mean arterial blood pressure (MAP) to describe the average response in blood pressure (i.e., MAP = [(2 × diastolic) + systolic]/3).

2.6. Statistical analysis

All statistical tests were carried out using SPSS version 15 (Windows). Statistical analysis of sampled cortisol, MAP, and heart rate (log-transformed) were subjected to repeated measures general linear models (GLMs), with time as the repeated measure, to evaluate effects of Sex (male and female), Genotype (val/val and val/met), Time (eight sampling time-points) and two- and three-way interactions between variables (e.g., Sex × BDNF; Sex × BDNF × Time). In addition to these analyses, univariate tests were applied to supplementary cortisol measures (AUCi; Pruessner et al., 2003) to ascertain reliability of findings. To test for Sex - × Genotype effects on blood pressure (MAP) and heart rate, data were subjected to multivariate GLMs (Sex × BDNF), with univariate follow-up tests to explain statistical interactions. Huynh—Feldt corrections were applied if sphericity (significant differences in variance between groups) was significant, and only adjusted results are reported.

3. Results

3.1. Salivary cortisol

3.1.1. Preliminary analysis

Since stage of the menstrual cycle has been reported to impact salivary cortisol rises in the TSST paradigm (Kirschbaum et al., 1999), we compared cortisol measures obtained from women who were using oral contraception (OC+) versus women who were not using oral contraception (OC—), and then we compared the cortisol measures of each group of women to men. None of the analyses yielded significant results; these included GLM repeated measure across the eight time-points (OC Group × Time: $F = 0.21$, $p = 0.646$, d.f. = 1), and univariate comparisons (by OC group) of AUCi ($F = 0.01$, $p = 0.901$, d.f. = 1) (Table 2). Additionally, both groups of women differed significantly from the men on the same measures (Table 2), and uniformly the values obtained from men were higher than those of the women. Based on these results, contraceptive-use was not considered in subsequent analyses. Additionally, a comparison of cortisol measures between the two main ethnic groups (Ashkenazi, Sephardic) yielded negative results.

3.1.2. Time-related changes in cortisol

Results were first analyzed by repeated measure GLM analysis of log-transformed cortisol levels (Sex × Time), with time as the repeated measure. There was a significant within-subject effect of time by sex interaction (GLM: $F = 11.52$, $p = 0.0002$, d.f. = 3.13, estimated effect size $\eta^2 = 0.11$). These results reflected the overall higher response of men at eight time-point scale compared to women (Fig. 1a). Next included in the repeated measure model was BDNF genotype. There was no significant effect of Time × BDNF (GLM: $p = 0.595$, d.f. = 3.33). However, as shown in Fig. 1b, tests of within-subject effects showed a significant three-way interaction

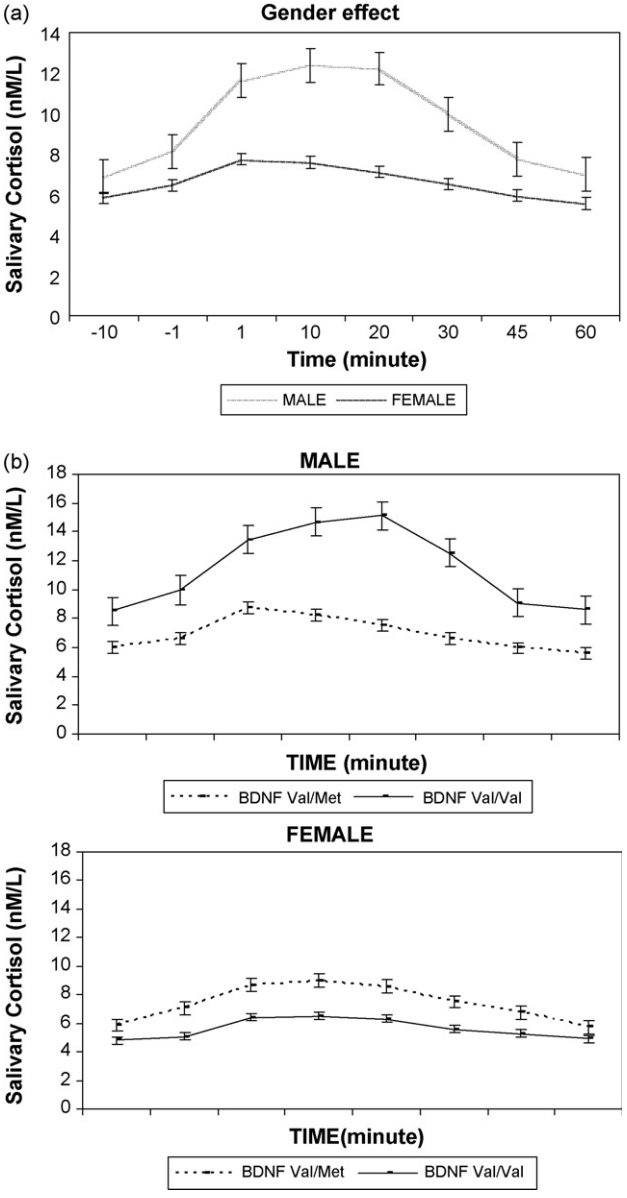

Figure 1 (a) Effect of sex and time on salivary cortisol values sampled during the TSST. (b) Modulation of salivary cortisol by BDNF val66met and sex during the TSST.

(GLM: Time × BDNF × Sex: $F = 6.15$, $p = 0.0002$, d.f. = 3.33). Notably, in male subjects, val/val homozygotes showed an overall larger response than val/met heterozygotes, which was significant when males only were analyzed (GLM: $F = 4.22$, $p = 0.007$, d.f. = 2.99). In contrast, for females a trend was observed for carriers of the val/val genotype, reflecting an overall lower response than the val/met group (GLM: $F = 2.18$, $p = 0.076$, d.f. = 3.84). We also examined AUCi regarding the allelic effects in women. A significant difference was observed in women between the val/val and val/met genotype (univariate: $F = 6.296$, $p = 0.016$, d.f. = 1; AUCi (SE): val/met = 133.58 (38.2), val/val = 20.65 (23.8)), which was opposite to the results observed in male subjects (see supplementary data, Fig. Ia). Sex and BDNF genotype explained 6% of the variance in individual differences in salivary cortisol ($\eta^2 = 0.06$).

3.2. Blood pressure and heart rate

With regards to MAP during the TSST test of within-subject effect showed a significant interaction between Time × Sex in the repeated measures GLM analysis ($F = 4.91$, $p = 0.004$, d.f. = 2.67), with men's responses being higher then women's responses at all time-points, and a significant between-subjects effect Time × BDNF × Sex interaction ($F = 5.30$, $p = 0.024$, d.f. = 1) (see supplementary data, Fig. IIa). For heart rate responses, the test for a between-subjects effect showed a significant interaction between Time × Sex in the repeated measures GLM analysis ($F = 7.35$, $p = 0.008$, d.f. = 1), with higher responses for females then males at all time-points. Tests of within-subject effects showed a significance interaction Time × BDNF × Sex ($F = 2.73$, $p = 0.044$, d.f. = 3) (see supplementary data, Fig. IIb).

3.3. Self-report measures of stress

At the end of the TSST, subjects were asked to rate their subjective feelings of stress during the testing procedure. The results showed an almost identical pattern as salivary cortisol, MAP, and heart rate measures. There was a significant Sex × BDNF interaction with self-reported stress as the dependent variable (univariate ANOVA: $F = 4.65$, $p = 0.034$, d.f. = 1) (see supplementary data, Fig. III). No main effect was evident for either BDNF or sex.

4. Discussion

The present results extend the findings of a small number of investigations indicating that common polymorphisms modulate the HPA axis response to laboratory-induced social stress using the TSST paradigm (Uhart et al., 2004; Wust et al., 2004; Chong et al., 2006; DeRijk et al., 2006; Gotlib et al., 2007). Beyond this, we report that the role of the BDNF Val66Met polymorphism on salivary cortisol levels showed contrasting effects in men and women. Overall, a similar pattern of results were found for the sympathetic measurements, which were significant for mean arterial blood pressure and heart rate. As such, the results concur with those of Kumsta et al. (2007), who reported significant sex-specific associations between the *GR* gene polymorphisms and HPA axis responses to psychosocial stress.

We note that the greater cortisol response in men compared to women is a potential confounding factor in interpreting allelic effects across sexes. It should be considered that the greater cortisol rise in men facilitates observing allelic differences whereas in women the lower overall cortisol response would tend to obscure such differences. Nevertheless, in the current investigation we have showed that women val/val homozygotes had lower response than val/met women which was significant with AUCi as the measure. In male subjects val/val homozygotes had higher responses than val/met subjects.

The sexual dimorphism observed in the current investigation is not atypical in the study of complex phenotypes. For example, in a recent publication, Weiss and colleagues examined quantitative traits that are associated with common human diseases, such as heart disease, hypertension, diabetes, asthma, and autoimmune disease (Weiss et al., 2006). Of the 17 traits that were surveyed, 11 were sexually dimorphic, and 12 showed evidence of differences between the sexes in heritability or linkage. These results suggest that many genes including BDNF, or the genetic variations within them, may act differently in males and females. Many of these gender effects may be the result of hormonal influences on gene expression and regulation or other non-genetic factors that are correlated with gender. For BDNF, the effect of estrogen on levels of this neurotrophin is well documented (Sohrabji and Lewis, 2006), perhaps providing the cellular milieu for the observed allele-specific genetic dimorphism reported in the present study.

As observed initially by Kirschbaum et al. (1992) and reviewed in Kudielka and Kirschbaum (2005), mean cortisol responses were 1.5- to 2-fold higher in men compared with women in the TSST, an apparently paradoxical finding since women are diagnosed with anxiety- and mood-related pathology at higher rates than men, with many epidemiological studies indicating that the female-to-male ratio is approximately 2:1 for mood and anxiety disorders (Gater et al., 1998). On the other hand, hypertension and other types of stress-related disorders are higher among men than women (Moller-Leimkuhler, 2007). The findings of Gender × Genotype interactions in stress responses can be an important lead in the examination of stress among men and women as well as individual differences in vulnerability to stress disorders within samples of men and women carrying different alleles.

We suggest that this apparent inconsistency (high anxiety and low cortisol response to social stress across clinical and non-clinical groups of women) may be informed by genetic information that sheds light on stress-related gender differences. We have shown that, dependent on genotype, men and women either markedly differ in salivary cortisol (val/val males versus val/val females) or revealed almost identical responses (val/met females versus val/met males) to acute social stress. An important take-home message from our investigation is that informative genotyping may help resolve paradoxical observations related to stress-related sex differences across clinical and non-clinical groups. Indeed, the development of depression is due to both stressful life events and modified by genotype (Caspi et al., 2003). Women with the BDNF Val66Met genotype and men with the Val66Val may be particularly vulnerable to social stress mediated by HPA axis activity.

The interaction of psychosocial stress, gender, and genes contributes to illness including cardiovascular disorders (Weidner and Cain, 2003), depression (Noble, 2005), diabetes type II (Legato et al., 2006), and PTSD (Olszewski and Varrasse, 2005). Depending on an individual's ability to mobilize both psychological and physiological coping mechanisms (stress resilience) (Southwick et al., 2005; O'Donnell et al., 2008), good health and wellness can be maintained despite the challenges. Moreover, stress is not only experienced at a single moment but also occurs across the life span from childhood onward and it is the accumulated effect of stress (allostasis) that challenges individual coping mechanisms (Turner-Cobb, 2005). Of particular importance in this research has been the elevation in levels of the hormone cortisol, the end product of the HPA axis, measured in saliva, as a reliable indicator of increased physiological stress arousal (Kirschbaum and Hellhammer, 1989). The sexes differ on a range of coping variables following stressful life events, e.g. cardiovascular events (Gulliksson et al., 2007), and the current investigation underscores the importance of specific polymorphisms in possibly shaping such sex differences.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen.2008.09.017.

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