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Cortisol and behavior in fragile X syndrome

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Abstract

Objective: The purpose of this study was to determine if children with fragile X syndrome, who typically demonstrate a neurobehavioral phenotype that includes social anxiety, withdrawal, and hyper-arousal, have increased levels of cortisol, a hormone associated with stress. The relevance of adrenocortical activity to the fragile X phenotype also was examined.

Method: One hundred and nine children with the fragile X full mutation (70 males and 39 females) and their unaffected siblings (51 males and 58 females) completed an in-home evaluation including a cognitive assessment and a structured social challenge task. Multiple samples of salivary cortisol were collected throughout the evaluation day and on two typical non-school days. Measures of the fragile X mental retardation (*FMR1*) gene, child intelligence, the quality of the home environment, parental psychopathology, and the effectiveness of educational and therapeutic services also were collected. Linear mixed-effects analyses were used to examine differences in cortisol associated with the fragile X diagnosis and gender (fixed effects) and to estimate individual subject and familial variation (random effects) in cortisol hormone levels. Hierarchical multiple regression analyses were conducted to determine whether adrenocortical activity is associated with behavior problems after controlling for significant genetic and environmental factors.

Results: Results showed that children with fragile X, especially males, had higher levels of salivary cortisol on typical days and during the evaluation. Highly significant family effects on salivary cortisol were detected, consistent with previous work documenting genetic and environmental influences on adrenocortical activity. Increased cortisol was significantly asso-

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ciated with behavior problems in boys and girls with fragile X but not in their unaffected siblings.

Conclusions: These results provide evidence that the function of the hypothalamic-pituitary-adrenal axis may have an independent association with behavioral problems in children with fragile X syndrome.

Keywords: Fragile X syndrome; Cortisol; Stress; Neuroendocrine; Behavior; FMRP

1. Introduction

Fragile X syndrome, caused by mutations in a single gene on the long arm of the X chromosome, occurs in 1 of every 2000 to 5000 live births and is the most common known inherited cause of developmental disability. The cytogenetic fragile site on the X chromosome from which the syndrome derives its name is typically caused by the presence of more than 200 cytosine-guanine-guanine (CGG) triplet repeats within the promoter region of the fragile X mental retardation (*FMR1*) gene, which prevents normal transcription. This "transcriptional silencing" of the gene and the subsequent diminished or absent production of the *FMR1* protein (FMRP) results in aberrant brain development and function (Devys et al., 1993; Tamanini et al., 1997). Because females have two X chromosomes, production of FMRP is maintained to varying degrees by the presence of the unaffected X chromosome. Consequently, females tend to be less severely affected by fragile X than males.

In addition to cognitive impairment, individuals with fragile X typically demonstrate a neurobehavioral phenotype that includes stress-related symptoms such as hyper-arousal, hyper-responsivity to sensory stimuli, hyperactivity, impulsivity, gaze aversion, and social anxiety and withdrawal (Cohen et al., 1988; Lachiewicz, 1992; Freund et al., 1993; Lachiewicz and Dawson, 1994; Cohen, 1995; Mazzocco et al., 1998). Recently, FMRP expression has been linked to some of these phenotypic characteristics of fragile X, including social withdrawal, anxiety and depression (Hessl et al., 2001). Despite the relatively consistent links between *FMR1* gene function and outcomes in fragile X, considerable variability in stress-related behavior problems exists, ranging from high levels of distress, often in novel social situations, to normal functioning. This variability can in part be explained by non-genetic factors, such as characteristics of the home environment and the effectiveness of educational and therapeutic services (Hessl et al., 2001). However, other individual characteristics of children or the environments in which they live may help to better account for these individual differences, leading to more effective methods of assessment and treatment of stress-related symptoms.

One such individual characteristic, the function of the hypothalamic-pituitary-adrenal (HPA) axis, may help to explain some of the variability in stress-related symptoms among children with fragile X. Regulation of the HPA axis is complex and involves feedback mechanisms occurring at the level of the hypothalamus, pituitary, hippocampus, and frontal cortex. This dynamic system is mediated through the secretion of adrenal glucocorticoid hormones, and is involved in the regulation of

physiological and behavioral responses to stress. Activity of the HPA axis is a component of normal coping (Gunnar, 1987; de Kloet et al., 1999). The HPA response to stress is adaptive in that it prepares the individual for dealing with the source of the stress, however chronic elevations or disruptions in the typical diurnal rhythm of cortisol can lead to medical problems associated with immune suppression (McEwen et al., 1997) and adverse effects on the brain that interfere with learning and memory (Sapolsky, 2000). In addition, while it has long been known that the experience of stress can cause an HPA response, recent evidence suggests that a genetic-biological predisposition to neuroendocrine reactivity can lead to abnormal behavioral responses to stressful stimuli (Bakshi and Kalin, 2000).

Neuroendocrine studies in fragile X syndrome implicate abnormalities in the hypothalamic-pituitary system. For example, precocious puberty and elevated gonadotrophin levels have been described in individuals with fragile X (Butler and Najjar, 1988; Moore et al., 1990). In an evaluation of hypothalamic-pituitary-thyroid (HPT) function in 12 males with fragile X, Bregman et al. (1990) reported normal levels of thyroid stimulating hormone (TSH) but a blunted TSH response to thyrotropin releasing hormone (TRH). Further, Loesch and colleagues (Loesch et al., 1995) found that despite a high rate of physical growth in the preadolescent period, individuals with fragile X show less pubertal growth compared to normal relatives. These investigators hypothesized that premature activation of the hypothalamic-pituitary-gonadal axis may be cause of growth impairment in individuals with fragile X. While these studies do not directly show hypothalamic-pituitary-*adrenal* dysfunction in fragile X, they demonstrate that disruption of hormonal processes mediated by the hypothalamus and pituitary may be affected.

Children with fragile X often have abnormally strong physiological and behavioral responses to physical and social stimuli, thereby increasing their levels of arousal and possibly stress. For example, Miller et al. (1999) used a laboratory paradigm to study electrodermal responses to auditory, visual, touch, vestibular, and olfactory stimuli to assess sympathetic nervous system activity in children and adults with fragile X. In this study, increased electrodermal response (EDR) to stimulation and lower rates of habituation to stimulation were found in fragile X as compared to age and gender matched control subjects. Boccia and Roberts (2000), utilizing spectral analysis of heart beat intervals, found that boys with fragile X had increased heart rate and lower parasympathetic activity during experimental challenge. Thus, the anxiety, behavioral distress, and gaze avoidance typically observed in individuals with fragile X may be due to sympathetic-adrenomedullary over-reactivity.

To date, no study has comprehensively examined HPA function in individuals with fragile X syndrome. We conducted a pilot assessment of salivary cortisol levels in 15 children with fragile X in comparison to a group of normally developing children (Wisbeck et al., 2000). These results provided preliminary evidence for increased adrenocortical activity in fragile X; however the small sample sizes and the comparison group limited the generalizability of these findings. In the current study, we assessed adrenocortical activity and its relation to behavior via measurement of salivary cortisol in a large group of children with the fragile X full mutation in comparison to their unaffected siblings. For each child, cortisol data were obtained

at 4 sample times on two typical weekend days and at 6 times during a home visit that included cognitive and social challenges. By choosing a sibling comparison group, we were able to examine the effect of fragile X syndrome on adrenocortical activity by comparing two groups of children who share similar environments and inheritance of HPA function (Wust et al., 2000), but who differ primarily by virtue of their fragile X status.

Based on the behavioral phenotype, as well as evidence of autonomic and behavioral over-reactivity and other hormonal abnormalities, we hypothesized that children with fragile X would have higher levels of cortisol in comparison to their unaffected siblings. We hypothesized that this difference would be present on typical days and during the home visit, but more pronounced during the cognitive and social challenges. We also predicted that males with fragile X would demonstrate higher levels of cortisol than females with fragile X, given the known gender differences in *FMR1* protein expression and phenotype severity (Tassone et al., 1999). Finally, we predicted that, after controlling for factors that significantly influence behavior in fragile X such as FMRP, intelligence, the effectiveness of educational and therapeutic services and parental psychopathology, increased adrenocortical activity would further contribute to the severity of behavior problems in these children.

2. Methods

2.1. Subjects

Subjects were 109 children with the fragile X full mutation (39 girls and 70 boys) and their unaffected siblings (58 girls and 51 boys). In families having more than one child with fragile X and/or more than one unaffected child, matched pairs were chosen based on age and gender when possible. Children were between 6 and 17 years of age (fragile X: $M = 10.82$, $SD = 2.83$; unaffected siblings: $M = 11.26$, $SD = 3.16$). The sample of children was 91.7% Caucasian, 2.5% Hispanic, 2.5% African American, 1.7% Asian, and .8% Pacific Islander, and .8% Multi-Ethnic. Fragile X diagnoses of all children were confirmed by Southern Blot DNA analysis as detailed by Taylor et al. (1994). Potential participants with current endocrine disorder, febrile illness, or those taking steroid medications (e.g. for asthma), and their matched sibling, were excluded from the analyses. Two-thirds of the boys with fragile X (66%) and over one-quarter of the girls with fragile X (28%) were taking medication at the time of the visit, in comparison to 8% of their unaffected siblings. For children with fragile X, these medications primarily included: stimulants (30% of the sample), antidepressants (20%), antihypertensives (10%), and antipsychotics (6%). Twenty-three percent of boys and 3% of girls with fragile X were taking more than one class of medication. Written informed consent was obtained from the parents of all participants. Assent was obtained when the children understood the procedure.

2.2. Measures and procedures

2.2.1. Evaluation day

On the morning of the evaluation day, each child with fragile X and his/her sibling completed a test of intelligence and three brief neuropsychological measures (word fluency, spatial relations, and executive functioning). In the afternoon, both children completed a structured social challenge (described below). Cortisol samples were collected 1) within 30 min of wakening and prior to breakfast (0742 h ± 36 m) during the testing (1100 h ± 4 m), 3) prior to the social challenge (1508 h ± 30 m), 4) 30 min. after the social challenge (1538 h ± 31 m), 5) 90 min after the social challenge (1639 h ± 30 m), and 6) at bedtime (2037 h ± 52 m).

The social challenge sessions were started at approximately 1500 h. Each child's session lasted 15 to 20 min and consisted of a structured child interview, a silent reading, an oral reading, and singing of three popular songs [modified version of a social behavioral challenge task (Herbert et al., 1991)]. Throughout the interview, children were asked to maintain eye contact as much as possible. For both reading tasks, non-reading children were given single words or letters. The order in which children with fragile X and their siblings completed the challenge was counterbalanced.

2.2.2. Typical days

Cortisol samples were collected by the parent 1) within 30 min of wakening and prior to breakfast (0810 h ± 65 m) one hour prior to lunch (1146 h ± 67 m), 3) one hour prior to dinner (1712 h ± 79 m), and 4) at bedtime (2035 h ± 123 m) on two consecutive non-school days. Parents were asked to choose "typical days," or days without the occurrence of unusually stressful or exciting events (e.g., birthday party, trip, doctor's appointment, sport or other performance).

The protocol for collection of the saliva sample was via a salivette roll (Sarstedt, Inc., Germany) soaked in the subject's mouth for about 1–2 min. To avoid contamination of saliva samples, parents were asked to forbid consumption of products containing citric acid at least 30 min and dairy products for at least 60 min prior to sampling (Magnano et al., 1989; Schwartz et al., 1998), to collect samples before children brushed their teeth, and to avoid days when children showed signs of illness.

2.2.3. Saliva preparation and assay

Salivettes were centrifuged at 4000 rpm for 5–7 min. The recovered saliva samples were then placed in a −20°C freezer until shipped to the assay laboratory (Fairview-University Medical Center Endocrine Laboratory, Minneapolis, MN). The samples were assayed in batches of 50, balanced for sex and group, with all samples from a fragile X subject and comparison sibling in the same batch to avoid introduction of error due to assay batch variation. The saliva was processed using the Magic Cortisol radioimmunoassay kit (Bayer, Tarrytown, NY) adapted for salivary cortisol assessment (Kirschbaum et al., 1989). The inter-assay coefficient of variation was 13.2% and the intra-assay coefficient of variation was 9%. Each sample was assayed twice, with duplicate correlations $> .95$. Cortisol levels were measured in micrograms/deciliter (μg/dl).

2.2.4. Intelligence

Children were administered the Wechsler Intelligence Scale for Children, Third Edition (Wechsler, 1991). The WISC-III is a well-standardized intellectual assessment for children ages 6 to 16 years.

2.2.5. Behavior problems

The Child Behavior Checklist (CBCL; Achenbach, 1991) is a parent-report, well standardized and widely used instrument, with several factors including withdrawn behavior, social problems, anxiety and depression, somatic complaints, attention problems, thought problems, aggressive behavior, delinquent behavior, as well as overall internalizing, externalizing, and total behavior problem scores.

2.2.6. Parental psychopathology

The Symptom Checklist—90-Revised (SCL-90-R; Derogatis, 1994) is a 90-item self-report of current psychological symptoms. The SCL-90-R yields nine primary symptom dimensions (somaticism, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism) and three global indices (global severity, positive symptom distress, and positive symptom total). Father and mother SCL-90-R scores within each family were averaged to create an overall composite. (Use of separate mother and father scores in the analyses would have reduced the sample to families with two parents). When father data was unavailable (15 of 109 or 14% of families), the parent psychopathology score was based on only the mother's score.

2.2.7. Home environment

The home environment was assessed using the Home Observation for Measurement of the Environment (HOME; Caldwell and Bradley, 1984). The HOME is a semi-structured interview and observation done in the family home. Factors include parent responsivity, encouragement of maturity in the child, acceptance of the child, learning materials present in the home, effort to provide cultural, recreational, or artistic enrichment, family companionship, and the quality of the physical environment of the home. For purposes of inter-rater reliability, two examiners made independent ratings of observational items on the HOME during visits of 22 homes. Then, approximately two weeks after the visit, one of the examiners (who tested the children and did not administer the parent interviews) contacted the parent by phone to administer the interview items. Inter-rater reliability for the HOME total score was high (intraclass correlation = 0.84).

2.2.8. Educational and therapeutic services

Due to a dearth of measures designed to assess the effectiveness of special education services, a new measure was developed for this purpose. The Special Curriculum Opportunity Rating Scale (SCORS; Dyer-Friedman et al., 2001) includes a 15 item Q-sort allowing the parent to rank the cognitive and behavioral skills that a child needs to develop. The parent then ranks the same 15 items according to how much the skills have actually improved in the past 6 months. The items include

academic, emotion management, planning, social, speech and language, and other skills needed for development. The correlation of the two 15 item Q-sorts is a measure of the effectiveness of a child's educational and therapeutic services to meet his or her current developmental needs. Test-retest reliability 1–2 weeks apart with 15 children was adequate for the developmental needs ($r = 0.69$) and improvement ($r = 0.68$) Q-sorts. Initial analyses demonstrate that the SCORS has good convergent and discriminant validity within this fragile X sample (SCORS; Dyer-Friedman et al., 2001).

2.3. Data analysis

To examine differences in salivary cortisol among boys and girls with fragile X and their siblings, and to account for individual and familial effects, we fitted linear mixed-effects models by restricted maximum likelihood (REML) using S-PLUS (Insightful, Seattle, WA). In addition to accounting for random effects (subject and family) and fixed effects (sample time, gender, and diagnosis), this method of analysis handles missing observations and unbalanced data sets more efficiently than a classic repeated measures ANOVA (Bagiella et al., 2000), allowing us to include all subjects who provided cortisol data. The response (dependent) variable was $\log_{10}$ salivary cortisol concentration. The $\log_{10}$ transformation was used to normalize the otherwise positively skewed distribution of cortisol values at each sample time. The fixed effects were sample time,[1] gender, and diagnosis (fragile X vs. sibling). We included gender by diagnosis and sample time by diagnosis interaction terms in the models to test differences in cortisol between males and females with fragile X and differences between groups in cortisol change across sample times. The random effects were subject (coded as a factor by each child's subject number) and family (each sibling pair was coded as a factor with the same family number). Subject was nested within family.

Next we examined the relation between adrenocortical activity and behavior in boys with fragile X, girls with fragile X, and comparison siblings. For purposes of data reduction, standardization across sample times, and reliability, we constructed two cortisol composite scores, one for the evaluation day (to represent cortisol response to cognitive and social challenge) and one for control days (to represent typical cortisol levels). For each composite score, cortisol levels were standardized by z-score transformation using the means and standard deviations of the sibling comparison group, and then combined by averaging. As stated earlier, we previously found that several environmental and genetic factors were associated with behavior

[1] Secretion of corticotrophin (ACTH) by the pituitary is episodic through the twenty-four hour cycle, and in response, basal cortisol secretion by the adrenal gland shows prominent circadian variation. The sample time factor accounts for this natural variation and allows for detection of differences in cortisol responses to task conditions. Given that cortisol response to a stressor is superimposed on the normal diurnal decline, significant changes in slope (i.e. a less steep decline) between subsequent sample times reflects a cortisol response to the intervening event. Using Helmert contrasts, each estimated time effect is the difference between the level of cortisol at a given time and the average of the previous levels.

problems in these three groups of children (Hessl et al., 2001). Therefore, in order to account for these factors and examine the remaining variance in behavior problems independently associated with adrenocortical activity, we utilized hierarchical linear regression analyses. For each group (comparison siblings, boys with fragile X, girls with fragile X), we entered factors from our previous model that were significantly associated with behavior problems in the first block[2] (for purposes here, nuisance variables) and the two salivary cortisol composites in the second block. Given limited samples sizes and statistical power concerns, we only included variables that were significantly associated with the outcome measures in each group.

3. Results

3.1. Random effects: individual and familial variation in salivary cortisol

To test for familial variation, we fitted models using subject as the only random effect, and compared them to models that also included the family effect using the likelihood ratio test. The addition of the family effect significantly improved the fit of the models on the evaluation day ($LR = 42.83$, $p < 0.0001$) and on the typical days ($LR = 51.08$, $p < 0.0001$). In fact, as can be seen by the estimates of the random effects (Tables 1 and 2), a greater proportion of the variance in cortisol was attributed to differences across families than individual subjects.

3.2. Fixed effects: fragile x- and gender-related differences in salivary cortisol

Fig. 1 illustrates the mean salivary cortisol levels ($\log_{10}$ transformed) of children with the fragile X full mutation in comparison to their unaffected siblings. Results of the mixed effects analysis for the typical days are shown in Table 1. Children with fragile X and their siblings demonstrated the normal diurnal decline in cortisol, showing significant decreases across sample times (all p's < 0.0001). A main effect of diagnosis ($t = -2.52$, $df = 102$, $p = 0.013$) and a time 2 by diagnosis interaction ($t = -2.06$, $df = 1316$, $p = 0.039$) showed that children with fragile X had higher levels of cortisol and a trajectory of cortisol levels across samples that deviated significantly from their siblings. A significant gender by diagnosis interaction ($t = -2.76$, $df = 102$, $p = 0.007$) confirmed that these differences were more prominent in males with fragile X. As can be seen in the figure, beginning with the Pre-Lunch sample, males with fragile X showed the highest levels of cortisol, and failed to show a normal decline in the evening.

On the evaluation day the four groups of children again showed the expected diurnal decline in cortisol (all p's < 0.0001). However, a significant diagnosis by

[2] For comparison siblings, these factors were gender, IQ, parent psychopathology (SCL-90-R), and the quality of the home environment (HOME). For boys with fragile X, factors were the effectiveness of educational and therapeutic services (SCORS) and parental psychopathology. For girls with fragile X, factors included IQ, parent psychopathology, and *FMR1* protein percentage.

Table 1
Salivary cortisol in children with fragile X in comparison to their unaffected siblings on typical non-school days: Mixed effects linear regression (REML) fit, parameter estimates, and probabilities[a]

Model Fit: AIC = 784.34, BIC = 853.60, LogLik = −379.17

Fixed effects:

Parameter	Estimate	Standard error	df	t	p
Intercept	−0.833	0.018	1316	−47.36	<0.0001
Time 2	−0.189	0.010	1316	−18.48	<0.0001
Time 3	−0.125	0.006	1316	−21.00	<0.0001
Time 4	−0.134	0.004	1316	−32.11	<0.0001
Gender	−0.001	0.010	102	−0.10	0.92
Diagnosis	−0.022	0.009	102	−2.52	0.013
Gender × Diagnosis	−0.030	0.011	102	−2.76	0.007
Time 2 × Diagnosis	−0.021	0.010	1316	−2.06	0.039
Time 3 × Diagnosis	−0.002	0.006	1316	−0.29	0.77
Time 4 × Diagnosis	−0.007	0.004	1316	−1.69	0.09

Random effects:

	95% Confidence Intervals		
	Lower	Estimate	Upper
Subject	0.033	0.060	0.109
Family	0.131	0.157	0.188
Within group error	0.272	0.283	0.294

[a] Notes: Time 2 = pre-lunch, Time 3 = pre-dinner, Time 4 = pre-bed. The time parameters show change in cortisol across sample times using Helmert contrasts. REML = restricted maximum likelihood. "Subject" is individual subject variation in salivary cortisol. "Family" is familial variation in cortisol across fragile X-sibling pairs.

time 2 interaction ($t = -3.01$, $df = 1002$, $p = 0.003$) showed that children with fragile X had less of a decline in cortisol between the pre-breakfast and cognitive testing periods (Fig. 1). Thus, the more gradual decline, observed predominantly in males with fragile X, represents increased cortisol reactivity in association with the experience of meeting the examiners and undergoing the cognitive evaluation. Following the cognitive testing period, males with fragile X continued to show cortisol elevation in comparison to their siblings throughout the social challenge, returning to levels of the other children at bedtime, following the departure of the experimenters.

In comparison to their siblings, many more children with fragile X, especially boys, were taking medications at the time of the assessment. To examine whether the cortisol increases observed in boys with fragile X were associated with medication use, we conducted two repeated measures analyses of variance (one for the evaluation day and one for typical days) with groups defined by current medication

Table 2
Salivary cortisol in children with fragile X in comparison to their unaffected siblings during an in-home evaluation: Mixed effects linear regression (REML) fit, parameter estimates, and probabilities[a]

Model fit: AIC = 696.00, BIC = 782.76, LogLik = −331.00

Fixed effects:

Parameter	Estimate	Standard error	df	t	p
Intercept	−0.849	0.022	1002	−37.86	<0.0001
Time 2	−0.204	0.013	1002	−15.16	<0.0001
Time 3	−0.089	0.008	1002	−11.70	<0.0001
Time 4	−0.046	0.005	1002	−8.66	<0.0001
Time 5	−0.042	0.004	1002	−10.22	<0.0001
Time 6	−0.084	0.003	1002	−24.07	<0.0001
Gender	0.021	0.015	106	1.32	0.191
Diagnosis	−0.018	0.012	106	−1.47	0.143
Gender × Diagnosis	−0.015	0.015	106	−1.01	0.316
Time 2 × Diagnosis	−0.040	0.013	1002	−3.01	0.003
Time 3 × Diagnosis	−0.003	0.008	1002	−0.35	0.727
Time 4 × Diagnosis	0.002	0.005	1002	0.31	0.758
Time 5 × Diagnosis	0.002	0.004	1002	−0.60	0.547
Time 6 × Diagnosis	−0.002	0.003	1002	−0.50	0.614

Random Effects:

	95% Confidence intervals		
	Lower	Estimate	Upper
Subject	0.102	0.130	0.164
Family	0.163	0.197	0.239
Within group error	0.256	0.270	0.279

[a] Notes: Time 2 = cognitive evaluation, Time 3 = pre-social challenge, Time 4 = post-social challenge (30 min), 5 = post-social challenge (90 min), 6 = pre-bed.

use (any versus none) as the between subject factor and $\log_{10}$ cortisol as the repeated measure. For typical days, neither a main effect of group [$F(1, 43) = 0.06$, $p = 0.81$] nor a group by time interaction [$F(5, 39) = 1.76$, $p = 0.14$] was found. For the evaluation day, boys taking medication had somewhat higher levels of cortisol at each sample time, however these differences did not reach significance, $F(1, 53) = 2.10$, $p = 0.15$. Regarding girls with fragile X, there was insufficient sample size in the medication group to complete this analysis.

3.3. Relation between salivary cortisol and behavior problems

Descriptive statistics of study variables are shown in Table 3. Detailed results and discussion of the factors initially found to be associated with behavior problems in

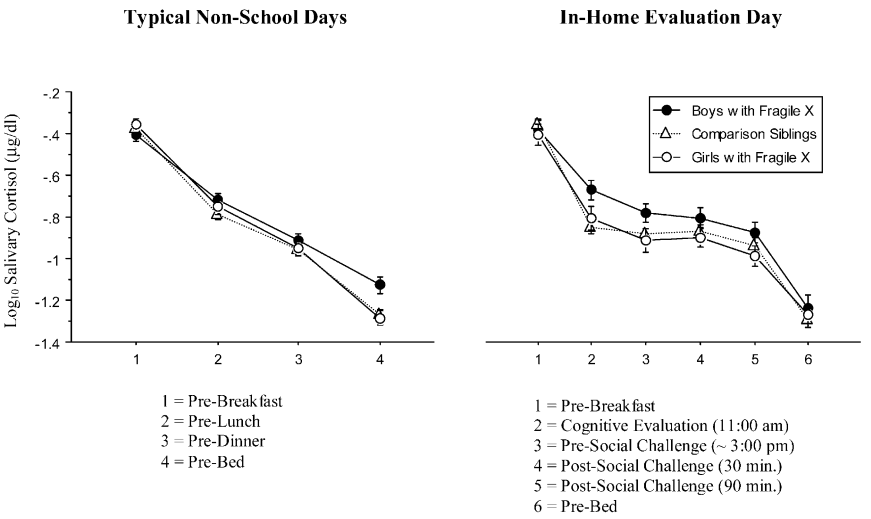

Fig. 1. Mean salivary cortisol levels (log10 transformed) of 39 females and 70 males with the fragile X full mutation and their unaffected siblings (58 females and 51 males) on 2 typical non-school days (collapsed across days) and an evaluation day. On typical days, a significant main effect of diagnosis ($t = -2.52$, $p = 0.013$) and a diagnosis by gender interaction ($t = -2.76$, $p = 0.007$) showed that, in comparison to their siblings, children with fragile X, especially males, had higher levels of salivary cortisol. On the evaluation day, a significant sample time by diagnosis interaction ($t = 3.01$, $p = 0.003$) showed that children with fragile X demonstrated increased cortisol reactivity to the experience of the cognitive evaluation and meeting unfamiliar experimenters. Error bars show standard errors.

this study are found in Hessl et al. (2001). Briefly, for boys with fragile X, less effective educational and therapeutic services and higher levels of parental psychopathology were predictive of behavior problems. For girls with fragile X, the results emphasized the influence of FMRP on internalizing problems and parental psychopathology on externalizing problems, while IQ was more generally associated with many types of problems. For comparison siblings, the factors significantly associated with behavior problems (IQ, home environment, and parental psychopathology) and levels of cortisol did not differ between girls and boys, however boys had higher behavior problem scores than girls [$t(107) = 2.31$, $p < 0.05$]. Therefore, we chose to combine the female and male sibling groups and include gender as one of the predictors in the following regression analyses.

For the unaffected siblings, results of the hierarchical regression analysis showed that after accounting for the quality of the home environment ($\beta = -0.22$, $p < 0.05$), parental psychopathology[3] ($\beta = 0.42$, $p < 0.001$), and non-significant effects of child

[3] The parent psychopathology variable is the mean of mother and father SCL-90-R scores. Although there was not a significant difference between mother and father scores [paired $t(93) = -0.53$, $p = 0.60$], the correlation between them was low [$r(92) = 0.16$, $p = 0.14$] indicating that they are independent. In families having one parent with high levels of psychopathology and the other with low levels, for example, the mean score could mask an important association between maternal or paternal psychopathology and child behavior problems. When the analyses were rerun using separate maternal and paternal SCL-90-R

Table 3
Descriptives statistics of variables by group[a]

	Control siblings (*n*=109)		Boys with Fragile X (*n*=70)		Girls with Fragile X (*n*=39)	
	Mean	SD	Mean	SD	Mean	SD
Age	11.26	3.16	11.04	2.64	10.41	3.14
IQ	108.66	11.95	45.98	7.36	76.13	22.20
FMR1 protein (%)	NA	NA	12.01	11.84	50.97	18.81
HOME environment	48.85	7.06	45.81	6.89	48.26	6.62
Effectiveness of services	NA	NA	0.22	0.41	0.40	0.33
Mother SCL-90-R			52.21	9.63	52.85	10.16
Father SCL-90-R			53.77	8.76	52.12	9.21
Salivary cortisol (μg/dl)						
Typical days						
Pre-breakfast	0.48	0.19	0.48	0.29	0.49	0.19
Pre-lunch	0.19	0.11	0.23	0.12	0.21	0.11
Pre-dinner	0.12	0.15	0.15	0.17	0.09	0.07
Pre-bed	0.08	0.14	0.14	0.25	0.06	0.05
Evaluation day						
Pre-breakfast	0.50	0.23	0.52	0.38	0.44	0.20
Cognitive testing	0.17	0.17	0.28	0.26	0.19	0.13
Pre-social challenge	0.16	0.17	0.22	0.22	0.14	0.09
Post-challenge (30 min.)	0.18	0.19	0.23	0.24	0.14	0.07
Post-challenge (90 min.)	0.16	0.21	0.19	0.23	0.11	0.06
Pre-bed	0.08	0.17	0.11	0.20	0.06	0.04
Child behavior checklist						
Internalizing problems	46.38	9.94	54.61	10.22	55.42	8.69
Externalizing problems	45.23	10.81	53.70	8.84	50.16	11.50
Total problems	43.77	12.05	61.09	11.23	56.26	11.23

[a] Notes: The HOME scores are derived from separate interviews about the environment of each child. Therefore, the quality of the home environment may differ between children with fragile X and their siblings.

IQ ($\beta = -11$, $p = 0.22$) and gender ($\beta = 0.14$, $p = 0.11$), salivary cortisol levels were not significantly associated with overall behavior problems (F change = 0.07, $p = 94$; total $R^2 = 0.36$, $p < 0.001$). For boys with fragile X, after controlling for the effectiveness of educational and therapeutic services ($\beta = -0.42$, $p < 0.01$) and parental psychopathology ($\beta = 0.23$, $p = 0.06$), the effect of cortisol approached significance, accounting for 8% of the variance in total behavior problems (F change = 2.67, $p = 0.07$; total $R^2 = 0.31$, $p < 0.001$). Increased cortisol levels were associated with greater severity of problems. Follow-up correlations with the CBCL subscales

scores (reduced sample size), the maternal, but not the paternal score was significantly associated with child problems in boys with fragile X ($\beta = 0.38$, $p < 0.01$) and in the comparison sample ($\beta = -0.36$ $p < 0.001$). For girls with fragile X, neither score was significantly associated with behavior problems. In considering these results, it is important to note that only mothers reported on child behavior problems.

showed that cortisol levels were most strongly associated with withdrawn behavior in this group [$r(58) = 0.28$, $p < 0.05$] such that higher levels of salivary cortisol were associated with more withdrawn behavior. For girls with fragile X, after removing effects of IQ ($\beta = -0.41$, $p < 0.01$), parental psychopathology ($\beta = 0.23$, $p = 0.11$), and FMRP ($\beta = -0.31$, $p < 0.05$), cortisol accounted for a significant proportion of the variance (14%) in total behavior problems (F change = 4.31, $p < 0.05$; total $R^2 = 0.53$, $p < 0.001$). Follow-up analyses revealed that cortisol level during the evaluation was significantly correlated with social problems [$r(39) = 0.42$, $p < 0.01$] and attention problems [$r(39) = 0.31$, $p = 0.05$]. Typical day cortisol was significantly correlated with attention problems [$r(38) = 0.41$, $p < 0.05$], while the correlation with somatic complaints [$r(38) = 0.30$, $p = 0.06$], and social problems [$r(38) = 0.29$, $p = 0.08$], approached significance. Fig. 2 shows the relation between salivary cortisol and total behavior problems in boys and girls with fragile X.

4. Discussion

Fragile X syndrome is a single gene disorder characterized by increased risk for a behavioral phenotype that includes social anxiety, gaze aversion, social withdrawal, and autistic behavior. In the current study, we found significant elevations in cortisol,

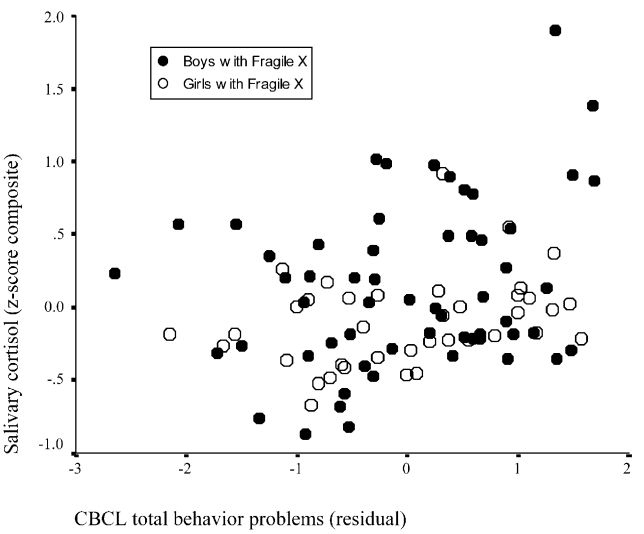

Fig. 2. Relation between salivary cortisol and CBCL total behavior problem residual scores in 58 boys and 39 girls with the fragile X full mutation. Hierarchical multiple regression analyses showed that, after removing effects of other significant factors, including IQ, FMR1 gene protein level, and the effectiveness of educational and therapeutic services, cortisol accounted for a significant proportion of the variance (8% and 14% in boys and girls, respectively) in total behavior problems such that increased levels were associated with greater severity of problems. Cortisol levels were standardized by z-score transformation and then averaged to derive the composite score. Residual scores represent behavior scores after the effects of the other independent variables in the multiple regression are removed.

a hormone of the HPA axis associated with stress, in children with fragile X in comparison to their unaffected biological siblings. The increases in cortisol were more pronounced in males, especially during conditions associated with cognitive testing and social demands. These findings replicate and extend previous results obtained from a different, much smaller sample (Wisbeck et al., 2000). In addition, we demonstrate the potential impact of increased cortisol in children with this diagnosis by showing that higher levels of this hormone were independently associated with behavior problems in boys and girls with fragile X (but not in unaffected siblings).

In total, utilizing several biological and environmental measures, we were able to account for over half the variance in behavior problems of girls and almost one-third the variance in behavior problems of boys with fragile X in these samples. The predictors of behavior covered several domains including child intelligence, *FMR1* gene function, and adrenocortical activity, as well as parental psychopathology, the home environment and the effectiveness of educational and therapeutic services. It is interesting to note that level of salivary cortisol predicted as much, or more of the variance in behavior problems as the level of protein expressed by the *FMR1* gene. Thus, the results highlight many sources of individual differences in behavior problems among children with fragile X, suggesting that multidimensional assessment may be necessary to best predict the outcomes of individual children or to describe the unique sources of behavior problems in different subgroups of children. On the other hand, our findings also demonstrate that a large proportion of the variance in behavior problems of children with fragile X, especially boys, remains unexplained. While a significant portion of this variance is certainly attributable to measurement error, other unknown characteristics of children and their families may be influential. For example, use and effectiveness of medication, parenting practices, the presence or absence of other siblings affected by fragile X, and other biological or genetic factors also may be associated with the frequency and severity of behavioral and psychiatric problems in these children.

The cross-sectional design of the study did not allow us to determine the causal relationship between adrenocortical activity and behavior in fragile X. Three models explaining this relationship are possible. First, the behavioral and emotional disturbances associated with the fragile X mutation may lead to an increase in cortisol. In this model, the decrement in FMRP causes neurodevelopmental changes in the brain that lead to phenotypic behavioral or psychiatric features such as social withdrawal, gaze avoidance, and anxiety. Stress-related affect and experiences, then, elicit increased adrenocortical activity. A second model emphasizes direct effects of increased cortisol on behavior. In this model, in normal conditions, FMRP has an effect on the physiological modulation of the stress response via its *direct* influence on the HPA axis and the autonomic nervous system. Thus, the FMRP deficit in fragile X would directly influence the functioning of the HPA axis, leading to the stress regulation difficulties that are characteristic of the syndrome. A third model, in which both of the above mechanisms are true, emphasizes that links between HPA axis activity and behavior are bi-directional, or continuously influencing one another.

Longitudinal studies are necessary to examine the trajectory and timing of behavior problems and adrenocortical activity over time.

Depending on the direction of causal links between adrenocortical activity and behavior, HPA function may play a role in the pathogenesis of the fragile X neurobehavioral phenotype. It is known that chronic elevations in cortisol are associated with alterations in neuronal development and morphology, especially in the hippocampus, due to a high concentration of glucocorticoid receptors in this region (McEwen, 1999). Paradoxically, while stress-related hippocampal atrophy is observed in animal studies and some human clinical samples (Sapolsky, 2000), *increased* hippocampal volume is observed in individuals with fragile X syndrome (Reiss et al., 1995; Kates et al., 1997). One explanation for this apparent incongruity is that the link between HPA dysfunction and brain function/structure in fragile X syndrome is complicated by the effects of diminished FMRP in the brain. To exemplify this point, increased density and morphological abnormalities of dendritic spines have been reported to occur in *FMR1* knockout mice (Comery et al., 1997; Weiler and Greenough, 1999) and in post mortem analysis of brain tissue from individuals with fragile X syndrome (Irwin et al., 2000), suggesting that the lack of the *FMR1* protein may interfere with normal synaptic pruning during neurodevelopment. Thus, any deleterious HPA effects on the hippocampus may be overshadowed by abnormalities in neuronal morphology associated with reduced FMRP. A longitudinal study of males with fragile X, especially those with relatively high levels of HPA activity, may in fact reveal atypical trajectories of hippocampal volume in association with persistent stress and stress reactivity. We are currently examining hippocampal function and structure, as well as HPA activity in a group of children with fragile X in an attempt to examine more closely, links between FMRP expression, neuroendocrine reactivity, brain function, and behavior.

In siblings unaffected by fragile X, no association between adrenocortical activity and behavior was found. There were clear differences between children with fragile X and their unaffected siblings in levels of cortisol and behavior problems. However variability in these measures was similar. This means that it is unlikely that the lack of association in the sibling group was due to limited variability. While there is a strong theoretical basis for a connection between cortisol and behavior in typically developing children, especially in regard to behaviors associated with inhibition, anxiety, fearfulness, and depression, empirical results have been inconsistent (Stansbury and Gunnar, 1994). Many studies linking adrenocortical activity to behavior in children have demonstrated that other variables such as attachment security, temperament, ego defenses, the presence or absence of a psychiatric condition, and social support can mediate the effects of stressful events on the adrenocortical system. Thus, links between adrenocortical activity and behavior in these children may exist, but only by taking into account one or more of these other characteristics. We believe that associations between cortisol and behavior were found in children with fragile X because of either a) the predominant effect of the syndrome on biological systems that *mediate* the perception of and response to stress or b) *direct* effects of the gene mutation on the HPA axis.

This study had limitations influencing the interpretation of the findings. First, the

average cortisol increase observed in children with fragile X, while statistically significant, was not equivalent to levels known to have negative effects on brain structure or cognitive function (Sapolsky, 2000). However, the stressors employed in this study were relatively mild. We do not know how the HPA axis in children with fragile X responds to higher levels and longer duration of stress, or the consequent effects on neural structure and function. Second, we cannot determine effects at higher levels of the axis, such as in brain regions having a high density of cortisol receptors. As has been hypothesized the adult literature, chronic persistent stress may eventually lead to HPA *under-reactivity* and *decreased* cortisol levels in some subjects, reflecting a physiological adaptation to stress in which less cortisol is needed because of increased receptor density or sensitivity over time (Yehuda et al., 1996; Stein et al., 1997). Thus, chronically stressed individuals, despite relatively low levels of unbound cortisol, may nevertheless be affected by the deleterious effects of HPA dysfunction. The present findings need to be followed by studies that allow assessment of the dynamics of HPA regulation. Third, the lack of a second comparison group of IQ-matched children without fragile X prevented us from ruling out the possibility that elevated cortisol was due to general effects of developmental disability. However, this is unlikely given the absence of a correlation between cortisol level and IQ in the fragile X group (data not shown). Fourth, the limited ethnic diversity of the sample limits the generalizability of these results to non-Caucasian children with fragile X. Finally, although parents were given detailed instructions regarding choice of "typical" days, consumption of foods that contaminate the assay, and recording the specific time of each sample, we were not able to directly monitor data collection on these days.

The results of this study, combined with previous evidence linking HPA function to alterations in brain structure, behavior, and cognition in other populations, suggest that assessment of this hormone system may be an important tool in understanding the neurobehavioral phenotype of fragile X. If the HPA axis does play a role in the development of the fragile X phenotype, pharmacological or environmental interventions designed to normalize HPA function might help to reduce stress-related problem behavior in affected individuals. Also, given that the internal experience of stress is often difficult to observe and that individuals with fragile X tend to underreport anxiety, HPA assessment may help to monitor their progress in treatment. Finally, the understanding of HPA function in a single gene disorder such as fragile X syndrome may provide insight into the neurodevelopment of other anxiety- or stress-related conditions.

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