Salivary Cortisol and Behavioral Response to Social Evaluative Threat in Adolescents with Autism Spectrum Disorder

E. Kale Edmiston, Scott D. Blain, and Blythe A. Corbett

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social behavior. One possible explanation for social communication deficits in ASD could be differences in biological systems that support responses to environmental stimuli. If so, it is unclear if differences in the arousal response to social stimuli in ASD are due to reduced interest in social information, or to an increased stress response. The hypothalamic pituitary adrenal axis facilitates arousal and the stress response to sensory input, including social stimuli. Previous research shows blunted cortisol response to social evaluative threat in children with ASD. The majority of prior work has focused on children with ASD, but adolescents with ASD are understudied. The adolescent period is of interest, as this developmental epoch is associated with increased salience of social evaluative threat in typically developing (TD) populations. In this study, we employed the Trier Social Stress Test (TSST), a laboratory paradigm that involves exposure to social evaluative threat, to study the cortisol and behavioral response to social evaluative threat in ASD and TD adolescents. Salivary cortisol data were collected at six time points before and after the TSST. Behavioral data were collected using video recordings of the TSST, which were then operationalized and coded. Paired sample t-tests were used to calculate within-group cortisol response to the TSST. Cortisol significantly increased in response to the TSST in the TD group but not the ASD group. The TD group showed a trend for more self-soothing behaviors during the stressor than the ASD group. The lack of a cortisol response to the TSST in the ASD group suggests that the TSST is not interpreted as stressful or salient for ASD adolescents. ***Autism Res*** *2016, 00: 000–000.* © 2016 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: autism spectrum disorder; adolescence; cortisol; HPA axis; displacement behavior; social evaluative threat

Introduction

ASD is characterized by alterations in social behavior, including impairment in social communication and diminished social insight [APA, 2013]. One possible explanation for social communication deficits in ASD could be differences in biological systems that support responses to environmental stimuli. If so, it is unclear if differences in the arousal response to social stimuli in ASD are due to reduced interest in social information, or heightened anxious arousal in response to perceived social evaluative threat. Because many individuals with ASD experience difficulty identifying or naming their own emotional states [Downs & Smith, 2004; Hill, Berthoz, & Frith, 2004; Griffin, Lombardo, & Auyeung, 2015], assessment of arousal and emotional state is challenging in this population. Recent work has found a correlation between physiologic arousal and self-report of enduring trait irritability [Mikita et al., 2015] as well as trait but not state anxiety [Simon & Corbett, 2013] in children with ASD, suggesting that insight into physiological arousal "in the moment" may be particularly challenging for this population, whereas reporting on stable trait characteristics is valid. Therefore, experimental approaches that assess for both physiological and behavioral indices of stress and arousal can provide a more objective view of the stress response in individuals with limited insight or ability to report on their subjective emotional state [Ostfeld-Etzion, Golan, Hirschler-Guttenberg, Zagoory-Sharon, & Feldman, 2015].

Cortisol is a neuroendocrine hormone that is the principal output of the hypothalamic-pituitary-adrenal (HPA) axis, the primary biologic facilitator of the stress response [Hennessy, Heybach, Vernikos, & Levine, 1979]. The HPA axis functions to facilitate adaptive responses to environmental stimuli, including social stimuli [Sapolsky, Romero, & Munck, 2000]. Cortisol is measurable in saliva, allowing for reliable, non-invasive sampling in children without the stress associated with blood sampling. By sampling saliva at baseline and at discrete intervals after a stressor, it is possible to

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measure the physiologic response to specific social contexts at an approximate 20-minute lag time [Kirschbaum & Hellhammer, 1999].

The majority of studies investigating salivary cortisol response to social stressors have employed the Trier Social Stress Test [TSST; Buske-Kirschbaum et al., 1997]. The TSST is a social evaluative threat behavioral paradigm that requires participants to give a brief speech about themselves in front of a neutral, unsupportive audience and is known to elicit a cortisol response in typically developing (TD) children [Buske-Kirschbaum et al., 1997], adolescents [Krishnaveni et al., 2014] and adults [Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999; Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004]. Previous studies employing the TSST in children with ASD have demonstrated a diminished cortisol response to social evaluative threat compared to TD children [Jansen et al., 2000; Lanni, Schupp, Simon, & Corbett, 2012; Levine et al., 2012]. Some studies have also found significant cortisol variability within children with ASD, suggesting possible subtypes who respond to social evaluative threat differently [Jansen, Gispen de Wied, van der Gaag, & van Engeland, 2003; Hollocks, Howlin, Papadopoulos, Khondoker, & Simonoff, 2014], attributed in part to degree of communication impairment [Jansen et al., 2003] or co-occurring social anxiety symptoms [Hollocks et al., 2014]. Thus, although the literature in pre-pubertal children suggests an overall blunting of the cortisol response to social evaluative threat, there may be heterogeneity within the ASD diagnosis that contributes to varying cortisol response profiles [Corbett et al., 2012].

Developmental factors may contribute to the salivary cortisol response to social evaluative threat in ASD. The majority of studies using the TSST in ASD have been with children; there has been one study of adults. This study found no differences in cortisol responsivity in adults with ASD compared to typically developing (TD) adults, implying that by adulthood, individuals with ASD demonstrate a comparable stress response to social evaluative threat [Jansen et al., 2006]. Thus, there may be a developmental process by which social evaluative threat becomes salient or stressful to individuals with ASD. To date, no studies have examined the relationship between salivary cortisol and social evaluative threat or social evaluative threat in pubertal adolescents with ASD. Adolescence is of interest in the study of social evaluative threat, as this developmental period is associated with the increased importance of social peers and an overall increase in complexity and nuance of the social world [Brown, Eicher, & Petrie, 1986; Roisman, Masten, Coatsworth, & Tellegen, 2004].

Adolescence is also important to the study of the stress response because the onset of puberty is known to impact the HPA axis [for review, see Romeo, 2010]. In TD populations, there are alterations of HPA axis response during puberty that impact the stress response [Di Luigi et al., 2006] and basal function [Barra, Silva, Rodrigues, Santos, & Colosimo, 2015]. There is a complex interplay between sex steroid hormones, which become elevated with pubertal onset, and the HPA axis [Payne, Kelch, Murono, & Kerlan, 1977]. For example, testosterone and estrogen both impact glucocorticoid production in the adrenal glands in non-human animals [Malendowicz and Mlynarczyk, 1982]. In rodents, testosterone also decreases plasma corticosteroid-binding globulin, the protein responsible for binding glucocorticoids, thereby increasing the amount of biologically active glucocorticoids [McCormick, Linkroum, Sallinen, & Miller, 2002]. As such, the rodent literature suggests an increase in the cortisol response to acute stressors in male adolescents compared to male adults [Goldman, Winget, Hollingshead, & Levine, 1973; Vázquez & Akil, 1993; Romeo, Lee, & McEwen, 2004; Romeo, Karatsoreos, & McEwen, 2006]. Studies in adult human males have also found an increase in testosterone levels that correlates with the cortisol response to the TSST [Lennartsson, Kushnir, Bergquist, & Jonsdottir, 2012; Bedgood, Boggiano, & Turan, 2014]. Furthermore, one study of male adolescents using a physical exercise stressor found that the magnitude of the cortisol response was correlated with pubertal stage, such that the greatest cortisol reactivity was found at the earliest stages of puberty [Di Luigi et al., 2006]. Overall, the extant literature in TD adolescents indicates a relationship between testosterone levels and cortisol reactivity to social stress. There is a large body of literature on the potential effects of exposure to prenatal sex steroid hormones, and conferred risk for the development of ASD [for review, see Romano, Cosentino, Laviola, & De Filippis, 2016], including evidence that fetal, but not postnatal, testosterone levels predict autistic traits in toddlers [Auyeung et al., 2012]. However, there is very little research identifying the impact of puberty on the stress response in ASD. Furthermore, many of the brain regions impacted by ASD, such as the hippocampus [Sussman et al., 2015], medial prefrontal cortex [Ernst, Zametkin, Matochik, Pascualvaca, & Cohen, 1997; Lombardo, Barnes, Wheelwright, & Baron-Cohen, 2007], and amygdala [Baron-Cohen et al., 2000; Schumann, Barnes, Lord, & Courchesne, 2009; Groen, Teluij, Buitelaar, & Tendolkar, 2010; Edmiston, Merkle, & Corbett, 2015], share reciprocal connections with the HPA axis [Cullinan, Helmreich, & Watson, 1996; Herman et al., 2003], and are known to undergo significant development during puberty [Giedd, 2004, 2008]. Taken together, these findings underscore a need for the study of the HPA axis response in pubertal adolescents with ASD.

In addition to salivary cortisol measures of stress reactivity, structured observational studies of behavior have been used in human and primate studies as an indirect measure of arousal [for review, see Troisi, 2002]. Behavioral observation methods, in conjunction with salivary cortisol, have also been employed in studies of children with ASD as a way to index duration and frequency of discrete behaviors [Schupp, Simon, & Corbett, 2013; Corbett et al., 2014]. For example, use of operationalized behavioral coding schemas to assess for social approach and avoidance behaviors has demonstrated diminished social-interactive play in children with ASD that correlates with physiologic arousal [Ostfeld-Etzion et al., 2015]. In studies of play behavior in children, individuals with ASD who engaged in more social behavior demonstrated a heightened cortisol response relative to those who engaged in less social behavior [Schupp et al., 2013]. Observation techniques can also be used to identify behaviors associated with heightened stress or arousal. For example, studies in human and non-human primates have identified a group of behaviors associated with the stress response, termed displacement behaviors [Troisi, 2002]. Displacement behaviors are self-directed activities such as self-grooming, hand-to-face contact, scratching, and lip and nail biting that have no specific communicative relevance to a context, but instead serve as distracters from stressful situations [Mohiyeddini, Bauer, & Semple, 2013]. Displacement behaviors are thought to be adaptive and serve as self-soothing or coping strategies that attenuate the stress response [Troisi, 2002; Mohiyeddini & Semple, 2013]. Indeed, this is supported by literature in rodents, where gnawing and chewing behavior in novel environments is associated with reduction in HPA axis activity [Hennessy & Foy, 1987; Levine & Levine, 1989]. Work in humans has also demonstrated a relationship between the frequency of displacement behaviors, such as self-grooming and hand-to-face contact, and biophysiological measures of stress, including heart rate and salivary cortisol following an acute social stressor [Sgoifo et al., 2003; Pico-Alfonso et al., 2007; Mohiyeddini et al., 2013]. However, the frequency of displacement behaviors has not yet been investigated in ASD.

Although the TSST is a commonly used behavioral paradigm for the assessment of physiological response to social evaluative threat in humans, no studies to date have operationalized or investigated stress behaviors during the TSST. There has been no investigation of the relationship between stress reactivity and behavior during the TSST or differences in frequencies of displacement behaviors during the TSST in ASD, and no research employing the TSST in a sample of pubertal adolescents with ASD.

The goals of the present study were to examine the HPA axis response to social evaluative threat in adolescents with ASD, as well as the behavioral correlates of stress and between-group differences in behavior during the TSST. We hypothesized that (a) ASD adolescents would show a blunted salivary cortisol response to the TSST and (b) there would be more variability in the ASD group compared to the TD group both at baseline and in response to the TSST. With regard to behavior, we anticipated that there would be (c) significant differences between the TD and ASD groups in the rate of stress behaviors observed during the TSST and that (d) variability in salivary cortisol during the TSST would correlate with the incidence of displacement behaviors during the TSST.

Methods

Participants

Twenty-eight participants with ASD (four females) and 18 age-matched participants with TD (three females) completed the study. The Vanderbilt University Institutional Review Board approved this study. Informed consent was obtained from parents prior to participation and assent was obtained from participants. Recruitment efforts included distribution of Institutional Review Board-approved flyers to university clinics, area schools, and resource centers, as well as the use of university and local autism listservs.

Procedure

The study required two visits to the university setting and three continuous days of home saliva sampling conducted between Visits 1 and 2.

Visit 1 consisted of diagnostic and neuropsychological testing, which included confirmation of ASD diagnosis, completion of study measures (outlined below), as well as instruction in home saliva sampling methods.

Wechsler abbreviated scale of intelligence [WASI, Wechsler, 1999]. All participants completed the WASI and had a Full-Scale IQ greater than 70.

The pubertal development scale [PDS, Petersen, Crockett, Richards, Boxer, et al. 1988]. The PDS is a parent report measure of physical development associated with puberty, including growth in height, body hair, skin changes/acne, and overall development compared to peers. The PDS includes voice deepening and facial hair questions for males and breast development and menarche questions for females. Likert scores range from 1: "change has not yet begun" to 4: "development

complete." All participants had a mean score of at least 2, indicating pubertal onset.

Autism diagnostic observations schedule version 2 [ADOS-2, Lord et al., 2012]. Adolescents with ASD completed the ADOS-2, Schedule 3 or 4, depending on developmental appropriateness, to confirm the presence of ASD. Research-reliable clinicians administered the ADOS. For first-time diagnoses, the Autism Diagnostic Interview-Revised [ADI-R, Lord, Rutter, & Le Couteur, 1994] was also administered to a parent. All ASD participants met ADOS-2 criteria for ASD with a total ADOS score greater than 7. Two participants who had ADOS-2 scores of 6 were included in the study and were determined to have ASD on the basis of developmental history, SCQ scores, ADI, and clinical judgment.

The social communication questionnaire (SCQ). The SCQ [Rutter, 2003], is a brief parent-report questionnaire that assesses for past and current behaviors indicative of ASD. In this study, the SCQ was used as a screening tool to rule out ASD in the TD group and to corroborate ASD diagnoses obtained via the ADOS. SCQ scores greater than or equal to 15 are thought to indicate ASD. No TD participant in the present study had a SCQ score greater than or equal to 10.

The social responsiveness scale second edition (SRS-2). The SRS-2 [Constantino & Gruber, 2012] is a parent-report questionnaire used to assess the presence of ASD symptoms across the following domains: Social Awareness, Social Motivation, Social Cognition, Social Communication, and Restrictive and Repetitive Behaviors. The standard SRS Total Score was calculated for each participant by summing the t-scores in each domain. The SRS identifies the severity of ASD symptoms and also can be used to differentiate social impairments in ASD from those that occur in other diagnoses. No TD participant had a SRS total t-score greater than 50.

Home sampling procedure

Our procedure for home saliva sampling is well-established and has been described in detail elsewhere [Corbett, Mendoza, Wegelin, Carmean, & Levine, 2008; Corbett et al., 2014]. Briefly, participants provided four samples per day on three continuous weekdays between Visit 1 and Visit 2, for a total of 12 at-home samples. Samples were collected at waking, 30 minutes post-waking, in the afternoon between 1:00 and 4:00 pm, and before bedtime. Participants passively drooled into a test tube through a straw, providing a minimum of one mL of saliva for each sample, kept refrigerated at home until Visit 2.

Trier Social Stress Test Paradigm

Visit 2 consisted of the TSST paradigm. At arrival, participants provided a saliva sample, followed by a second sample 20 minutes later. Lab personnel then directed the participant to the TSST room, where they were met by two novel raters wearing white lab coats and holding clipboards, one male and one female. Rater 1 was always an adult, and Rater 2 was an age- and gender-matched peer. Rater 1 provided instruction for the task, indicating that the participant had five minutes to prepare a speech, describing why they were the best candidate for a job. Following five minutes of preparation, the participant returned to the TSST room, where they spoke for five minutes. Raters did not provide feedback and maintained a neutral expression throughout. After five minutes, the participant was told to perform serial subtraction aloud, after which the raters debriefed the participant, assuring the participant that the task was "just pretend." The entire TSST protocol was videotaped by a camera placed on a tripod behind the raters to allow for analysis of behavior. Following the TSST, salivary cortisol samples were obtained by familiar lab personnel, one immediately after the paradigm, one 10 minutes after that, one 20 minutes following the paradigm, and a final sample 40 minutes following the end of the TSST, or 60 minutes after the beginning of the paradigm (see Fig. 1). In total, six samples were taken on the day of the TSST.

Additionally, as part of a larger study of autonomic nervous system regulatory capacity (Edmiston et al., in revision), participants completed a three-minute baseline electrocardiogram prior to the TSST, between collection of the first and second baseline saliva samples. Each participants' ECG data were cleaned and used to calculate mean respiratory sinus arrhythmia (RSA), an indirect measure of parasympathetic nervous system function, using heart rate variability software provided by Mindware Technologies (HRV 3.1, Gahanna, Ohio).

Immediately following the TSST, participants completed the **State-Trait Anxiety Inventory for Children** [STAIC, Spielberger, 1973]. Both the state and trait versions of the STAIC are 20-item questionnaires that are designed to measure anxiety symptoms in children. The state form measures acute anxiety, while the trait form measures features of anxiety as stable personality characteristics or temperamental traits.

Behavioral Coding Schema

Observer XT Version 8.0 software (Noldus, The Observer XT, 2008) was used for the analysis of behavioral coding of observational data. Behavioral coding data were analyzed based on established methods for determining frequency of specific behaviors operationalized in the lab [Corbett et al., 2014]. Briefly, the frequency of the

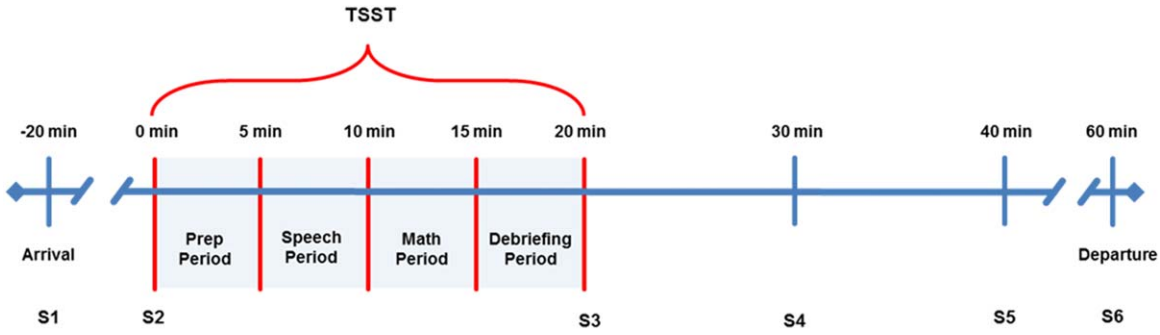

Figure 1. Schematic of TSST and salivary cortisol sampling procedure S1 = Sample 1

following behaviors was counted during one-minute intervals for the speech portion of the TSST: Displacement Behaviors (including Face Touch, Lip Press or Bite, Hand Fumble, and Grooming), Fidgeting, and Smiling (for operationalized description of behaviors, see Table 1). Inter-rater reliability was calculated for each behavior on a random sample of ten participants. Inter-rater reliability was established by calculating Cohen's Kappa, which controls for the possibility of raters agreeing by chance; for the present study, raters (SDB and EKE) established reliability at $K = 0.80$.

Salivary cortisol assay

The salivary cortisol assay was performed using a Coat-A-Count® radioimmunoassay kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) modified to accommodate lower levels of cortisol in human saliva relative to plasma. Saliva samples, which had been stored at −20 °C, were thawed and centrifuged at 3460 rpm for 15 min to separate the aqueous component from mucins and other suspended particles. All samples were duplicated. The coated tube from the kit was substituted with a glass tube into which 100 µl of saliva, 100 µl of cortisol antibody (courtesy of Wendell Nicholson, Vanderbilt University, Nashville, TN), and 100 µl of ^{125}I-cortisol were mixed. After incubation at 4 °C for 24 h, 100 µl of normal rat serum in 0.1% PO4/EDTA buffer (1:50) and precipitating reagent (PR81) were added. The mixture was centrifuged at 3460 rpm for 30 min, decanted, and counted. Serial dilution of samples indicated a linearity of 0.99. Interassay coefficient of variation was 1.62%.

Statistical analysis plan

Demographics and neuropsychological measures. Independent-group Student's T-tests were used to test for significant differences between the ASD and TD participants with regard to age, pubertal development, IQ, SCQ, and SRS scores, as well as state and trait versions of the STAIC.

Cortisol. Descriptive statistics showed positive skewness in the salivary cortisol data. All data were log transformed (base e) to align with the relative normality assumptions of ANOVA [Richdale & Prior, 1992; Corbett, Mendoza, Abdullah, Wegelin, & Levine, 2006; Lanni et al., 2012].

Levene's Test for Homogeneity of Variances was performed to determine if there were differences in variability between groups in salivary cortisol measures during the TSST.

ANOVA was performed to determine if the baseline cortisol values (mean afternoon over three days of home sampling: first sample taken at arrival on the day of the TSST, and second sample taken 20 minutes post-arrival), were comparable between the ASD and TD groups.

In order to determine if there was a physiological response to the TSST in each diagnostic group, paired sample *t*-tests were performed separately on the log-transformed ASD and TD samples using the sample taken immediately before the TSST and the sample taken immediately after the TSST ended. Main effects were considered significant at $P < 0.05$.

Repeated measures ANCOVA was used to analyze diagnostic effects on salivary cortisol during the TSST. We tested for between-group diagnostic effects with the salivary cortisol values at each of the four time points after the TSST as repeated measures. Mean afternoon basal salivary cortisol from the three days of home sampling was included as a covariate of no interest in the model. Results were considered significant at $P < 0.05$.

Pearson bivariate correlation was performed to assess for the relationship between baseline RSA and baseline salivary cortisol levels. Results were considered significant at $P < 0.05$.

Stress behaviors. To assess for group-wise differences in the frequency of coded stress behaviors, we employed Poisson regressions with diagnosis as the fixed factor for the following stress behaviors: Displacement Behaviors, Fidgeting, and Smiling (Table 1).

Table 1. Operationalized Stress Behaviors

Construct name		Operationalized Description
Displacement Behaviors	Face Contact	Any hand contact with face (i.e., rests hand on chin, scratches or rubs face, hand to mouth contact)
	Fumble	Repetitive motion of fingers or hands (i.e., tapping, twisting, wringing, or clenching)
	Grooming	Adjustment to hair or clothing to improve appearance (i.e., straightens collar or hem, runs fingers through hair)
	Lip Press or Bite	Lip movement not related to speech production (i.e., lip licking, biting, smacking, or pressing)
Fidgeting		Non-sustained movement of torso or extremities (i.e., swaying, bouncing, flailing)
Smiling		Facial expression resembling a smile (i.e., spontaneous social smiling or "forced" submissive/appeasement smile)

Table 2. Participant Demographics

Group (N)	Age (SD)	PDS score (SD)	VIQ (SD)	PIQ (SD)	FSIQ (SD)	SCQ total score (SD)	SRS total *T*-score (SD)	STAIC trait total (SD)	STAIC state total (SD)
TD (14)	14.99 (1.52)	2.89 (0.53)	113.43 (16.04)	110.93 (10.03)	113.86 (13.44)	2.29 (2.09)	42.43 (3.72)	31.55 (5.82)	31.91 (3.75)
ASD (24)	14.80 (1.36)	2.95 (0.49)	106.13 (23.12)	107.50 (18.44)	107.71 (22.03)	20.00 (9.91)[a]	74.88 (11.02)[a]	36.65 (5.99)[b]	32.55 (6.31)

VIQ, verbal IQ; PIQ, performance IQ; FSIQ, full scale IQ; SCQ, social communication questionnaire; SRS, social responsiveness scale; STAIC, state-trait anxiety inventory for children;

[a] $P < 0.0001$,

[b] $P < 0.05$.

Exploratory analyses were performed for each of the four component behaviors that comprise the Displacement Behaviors variable (Face Touch, Lip Press or Bite, Hand Fumble, and Grooming). All results were considered significant with $P < 0.05$.

Exploratory bivariate correlation analyses were performed within each diagnostic group to determine relationships between salivary cortisol levels during the speech portion of the task and at baseline and frequency of Displacement Behaviors. All results were considered significant at $P < 0.05$.

Results

Levene's Test demonstrated significant sex differences in cortisol variability during the stressor in the ASD group, with ASD females showing more variability than ASD males ($F = 11.757$, $P = 0.002$). Because we were underpowered to detect significant sex by diagnosis interaction effects, we removed the seven females from the sample and performed all further analyses on the male sample only. Levene's Test in the male sample indicated no significant differences between groups in cortisol sample variability (all P values > 0.50). ANOVA showed no significant between-group differences in baseline cortisol values (mean afternoon cortisol, cortisol upon arrival, or cortisol 20 min post-arrival ($F = 1.668$, $P = 0.205$).

There were no significant differences in age, pubertal development, verbal, performance, or full scale IQ between groups. There were, as expected, significant differences in SCQ and SRS scores between groups. There was no significant difference in self-reported anxiety post-TSST via the STAIC state measure between groups. There was, however, a significant difference in participant self-reported trait anxiety following the TSST (see Table 2).

Paired sample t-tests of the immediately pre- and immediately post-TSST cortisol values indicated a significant increase in cortisol in the TD group ($t = 3.296$, $P = 0.006$) and a non-significant trend in the ASD group ($t = 2.005$, $P = 0.057$, see Fig. 2).

Repeated measures ANOVA revealed non-significant differences between diagnostic groups in salivary cortisol across the four post-TSST samples when controlling for mean home basal cortisol levels ($F = 0.404$, $P = 0.529$). There was a significant main effect of time on cortisol values ($F = 14.723$, $P < 0.0001$) but no group by time interaction effects ($F = 0.487$, $P = 0.692$, see Fig. 3).

Descriptive statistics revealed extremely low counts of all behaviors during the serial subtraction portion of the TSST. As a result, we performed all statistical analyses on behaviors only during the speech portion of the TSST, where there was sufficient incidence of behaviors for statistical inference. Descriptive statistics showed a clear outlier for the Smiling Behavior code, with one ASD participant showing frequency of Smiling Behavior more than three standard deviations greater than the mean. This participant was removed from the sample for further analysis of the Smiling Behavior code. Subsequently, Poisson regression did not indicate a significant difference in rates of Smiling (chi square = 1.101, $P = 0.294$) or Fidgeting (chi square = 2.621, $P = 0.105$) between groups during the speech portion of the TSST (see Fig. 4).

There were marginally significant between-group differences in rates of Displacement behaviors during the speech portion of the TSST (chi square = 3.605, $P = 0.058$), with greater frequencies of Displacement behaviors in the TD group. Exploratory post hoc analyses of the component behaviors of the Displacement behavior variable showed significant between-group differences in rates of Lip Press or Bite (chi square = 4.407, $P = 0.036$), but not Hand Fumbling (chi square = 0.971, $P = 0.324$), Face Contact (chi square = 2.491, $P = 0.115$), or Grooming (chi square = 2.018, $P = 0.155$, see Fig. 4).

Bivariate correlation analyses within each diagnostic group indicated a significant negative correlation between Displacement Behaviors and cortisol values during the speech in the TD group ($r = -0.556$, $P = 0.048$), but not the ASD group ($r = -0.012$, $P = 0.959$, see Fig. 5).

Pearson bivariate correlation analysis between baseline RSA and baseline salivary cortisol in the total sample demonstrated a significant negative correlation ($r = -0.487$, $P = 0.005$, see Fig. 6).

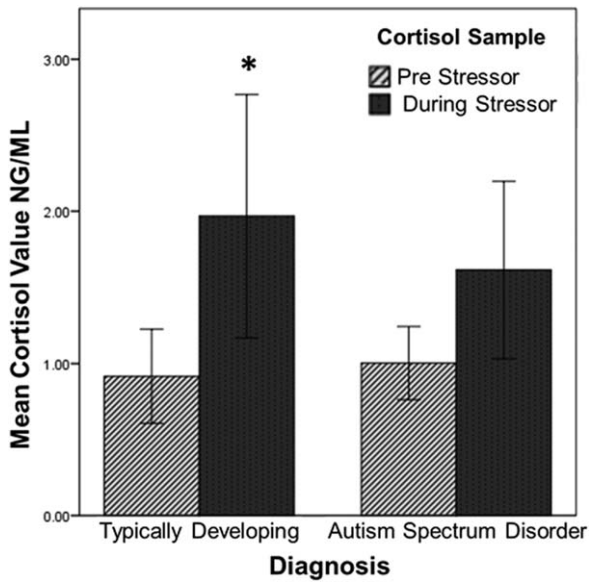

Figure 2. Within-group cortisol response to the TSST. Raw cortisol values depicted for ease of interpretation; all analyses performed on log-transformed data. Error bars represent SE.

Discussion

We aimed to determine the effects of social evaluative threat on HPA axis responsivity and behavior in adolescents with ASD. As expected, within-group comparison found that for the TD group, the immediate onset of the TSST was associated with a significant increase in cortisol. This increase in cortisol at the onset of the TSST revealed a non-significant trend in the ASD group. Repeated measures ANOVA analysis of the four samples taken post-TSST did not show any between-group differences in cortisol response curve. Although negative findings should be interpreted with caution give our small sample size, these results could suggest that the immediate response to the onset of the TSST is not present in ASD adolescents as it is in TD adolescents, but that there are no overall between-diagnosis differences in the maintenance of the stress response. Our findings are situated in a broader literature that shows atypical arousal in ASD, including work that shows altered sympathetic [Kushki et al., 2013; Neuhaus, Bernier, & Beauchaine, 2015], and parasympathetic function both at basal levels [Tomarken, Han, & Corbett, 2015] and in response to stressors [Guy, Sounders, Bradstreet, DeLussey, & Herrington, 2014; Ming, Patel, Kang, Chokroverty, & Julu, 2016].

There was a negative correlation between baseline cortisol on the day of the TSST and baseline RSA in both the ASD and TD groups [Edmiston et al., in press], indicating, as expected, that high basal PNS function is associated with reduced HPA axis output. To the best of

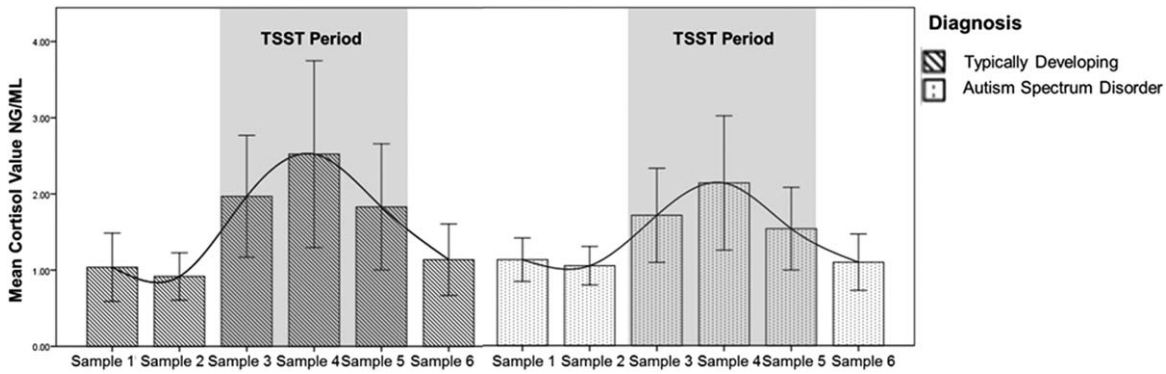

Figure 3. Salivary cortisol during the TSST in ASD and TD adolescents. Raw cortisol values depicted for ease of interpretation; all analyses performed on log-transformed data. Error bars represent SE.

our knowledge, this is the first study to demonstrate this relationship between ANS and HPA activity for individuals with ASD. The inverse relationship between RSA and salivary cortisol extends previous findings in TD adults [Cărnuţă, Crişan, Vulturar, Opre, & Miu, 2015], but not adolescents [Oldehinkel et al., 2011]. Heterogeneity in findings may be due to differing degrees of pubertal development across samples (i.e., peripubertal vs. pubertal), or differing maturational trajectories of the ANS versus the HPA axis [van den Bos & Westernberg, 2015].

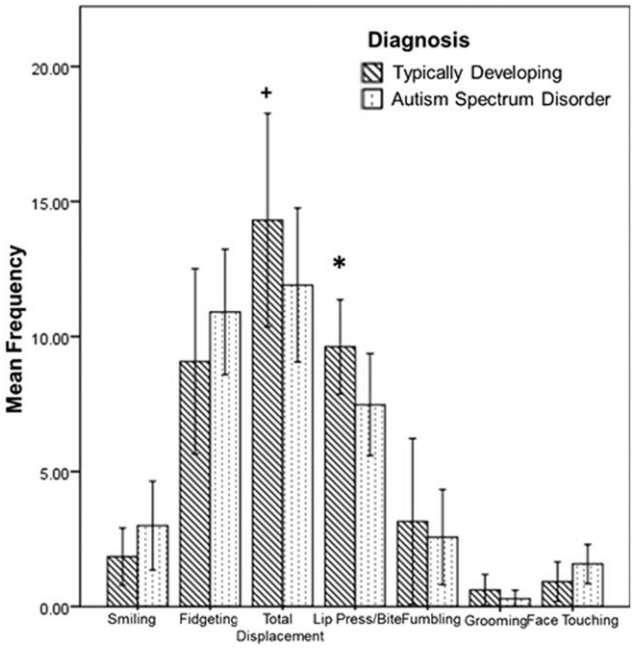

Figure 4. Between-group behavior frequencies during the speech portion of the TSST. $+P = 0.058$, $*P < 0.05$. Error bars represent SE.

Our findings largely substantiate previous work indicating a blunted salivary cortisol response to the TSST in children with ASD [Jansen et al., 2000; Lanni et al., 2012; Levine et al., 2012]. In one study by Lanni et al. [2012], children with ASD did not demonstrate a salivary cortisol response to the TSST, unlike TD children who mounted a significant stress response to the social evaluative threat task. The authors found a reduced cortisol response to the TSST but no between-group differences in subjective self-report of anxiety post-stressor, which coincides with the findings of this study. Given the high rates of anxiety within ASD [van Steense, Bögels, & Perrin, 2011], the reduced cortisol response to social evaluative threat in the present study could be caused by hypocortisolemia secondary to chronic stress exposure or anxiety; prolonged stress is known to attenuate cortisol production in adults [Martí and Armario, 1997; McEwen, 2004]. However, evidence from rodent literature suggests that there may be a paradoxical effect in adolescence, such that chronic stress results in an increased peak cortisol response [Romeo et al., 2006]. It is unclear what effect chronic stress may have on HPA axis function in TD or ASD adolescents.

Due to the small sample size, and non-significant cortisol trend in the adolescents with ASD in the current study, it is still important to consider plausible maturational cortisol effects. The only study to examine physiological reactivity to the TSST in adults with ASD and TD found no differences between diagnostic groups with regard to cortisol response, although the authors do

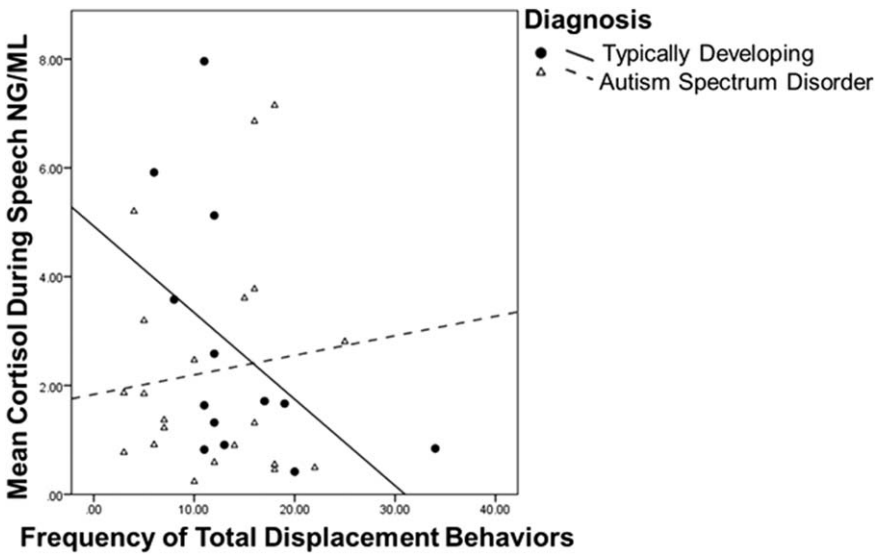

Figure 5. Correlation between displacement behaviors and salivary cortisol during the speech portion of the TSST.

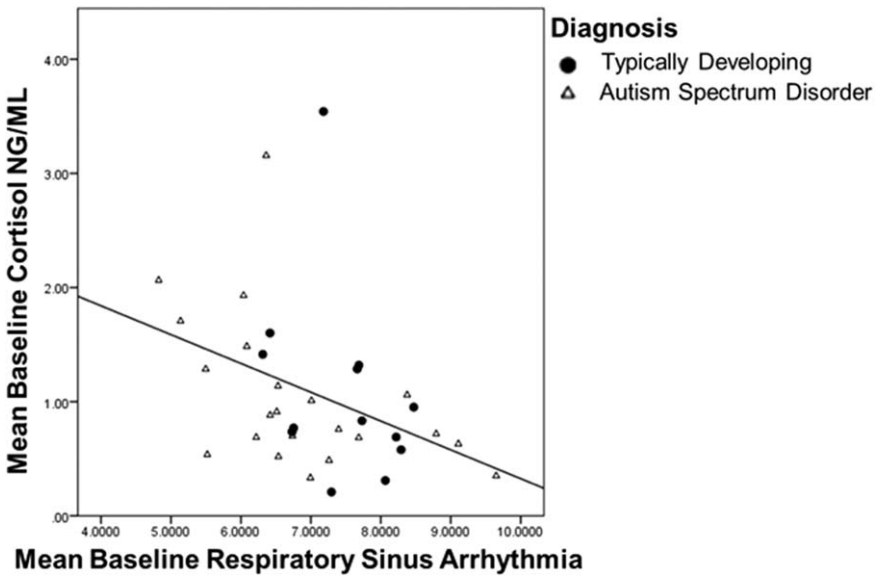

Figure 6. Correlation between baseline RSA and salivary cortisol measures.

report a blunted heart rate response in the ASD group compared to the TD group [Jansen et al., 2006]. When coupled with the findings of the present study, this could indicate a developmental effect such that, by adulthood, individuals with ASD demonstrate a typical stress response to social evaluative threat that is not present in childhood or adolescence. However, there are several methodological differences between the present study and Jansen and colleagues' work that could also account for the differences in findings, including differences in the sample composition and diagnostic criteria, as well as the use of a screen in the Jansen and colleagues' study that blocked the raters from view during the speech (2006). Clearly, longitudinal studies are needed to best determine the developmental effects on physiological reactivity to social evaluative threat in ASD.

Peer bullying and victimization are common challenges for ASD adolescents [Schroeder, Cappadocia, Bebko, Pepler, & Weiss, 2014; Zablotsky, Bradshaw, Anderson, & Law, 2014; De la Iglesia & Olivar, 2015; Fisher & Taylor, 2016; Weiss, Cappadocia, Tint, & Pepler, 2015]. As individuals with ASD enter adolescence, they may develop more insight into social marginalization and bullying, thereby impacting the stress response. Likewise, an emerging literature suggests that social marginalization and experiences with peer bullying are environmental factors that may have long-term consequences for HPA axis function. One such study has associated cortisol responsivity during the TSST with degree of social support in another group know to experience a great deal of peer bullying, lesbian, gay, and bisexual young adults [Burton, Bonanno, & Hatzenbuehler, 2014; Juster et al., 2015]. Social support can impact the HPA axis response to stressors, especially among adolescents that experience social exclusion or bullying [Hostinar & Gunnar 2015]. A recent study that altered the degree of status and acceptance threat during a modified TSST in healthy young adults found a greater cortisol response in situations with a greater degree of threat to social acceptance [Smith & Jordan, 2015]. This suggests that awareness of social status or concerns about social marginalization or inclusion can independently impact the cortisol response to the TSST and that there may be some relationship between cortisol responsiveness and social insight. Particularly given the great degree of variability in both cortisol responsiveness [Jansen et al., 2003; Hollocks et al., 2014] and social insight or theory of mind abilities in ASD [van Roekel, Scholte, & Didden, 2010], future studies should assess for the impact of social stigma on individuals' perception and physiological response to peer social evaluative threat, perhaps by assessing for level of insight into peer bullying [Zablotsky et al., 2014], or more general theory of mind abilities.

It is possible that for individuals with ASD, social evaluative threat in a structured setting such as the TSST is not interpreted as stressful. Because the requirements of the TSST are relatively clear, individuals with ASD may not interpret the task as stressful, as opposed to the more open-ended social situations they encounter in day-to-day life which combine unpredictability with novelty. For example, studies that assess for cortisol response to a naturalistic play situation with two other children found a heightened cortisol response to this relatively benign social interaction in ASD children compared to TD children [Corbett et al., 2010, 2012, 2014; Schupp et al., 2013] and other studies have found a heightened response to play with an unfamiliar peer in ASD [Lopata, Volker, Putnam, Thomeer, & Nida,

2008] and to the strange situation task in toddlers with ASD [Naber et al., 2007]. Furthermore, for children with ASD, the TSST may not be interpreted as a social stressor, but rather as a cognitive task; the neutral facial expressions of the raters in the TSST may not be as salient as the cognitive demands of giving a speech and correctly completing the serial subtraction task for individuals with ASD [Schultz, 2005; Krysko & Rutherford, 2009], or individuals with ASD may not interpret the neutral facial expressions of the raters as threatening due to differences in theory of mind or social perceptual abilities. Future studies in ASD populations should allow for comparison of social stressors that vary in their novelty and predictability, or assess for individual differences in insight into social exclusion, theory of mind ability and their relationship to cortisol reactivity to social evaluative threat.

With regard to behavior, there was a non-significant trend towards a greater number of Displacement Behaviors in the TD group that was driven by the greater frequency of the Lip Press or Bite Behavior. We did not find any other behaviors that approached significant difference between groups, but our small sample size limits our interpretation of the more modest behavioral findings in the present study. There was a significant correlation between Displacement behavior counts and cortisol during the speech portion of the TSST in the TD adolescents, such that lower cortisol values during the speech were associated with more Displacement behaviors. The relationship between Displacement behaviors and cortisol was not present in the ASD adolescents. These findings, although preliminary and exploratory, suggest that for TD adolescents, displacement behaviors are a self-soothing strategy during times of stress, and are therefore adaptive. Based on the data from our preliminary, exploratory analyses, this does not appear to be true for adolescents with ASD, although more research with larger samples is warranted.

Although ASD symptomatology was well-characterized in the present investigation, we did not formally assess for co-occurring anxiety disorders, and the ASD sample reported more trait anxiety than the TD group. However, parental reports of anxiety symptoms did not suggest an effect on cortisol response or behavior. One study that compared children with TD to children with ASD and either low trait anxiety or high trait anxiety found that altered physiological responsiveness to stress differentiated the high anxiety group in terms of behavior from both the TD group and the low anxiety ASD group [Panju, Brian, Dupuis, Anagnostou, & Kushki, 2015]. Future studies should examine the role of anxiety disorders in ASD on HPA axis function. Although this study was not designed to address causal mechanisms for differences in cortisol responsivity, future studies should examine potential mechanisms for the blunted stress response in ASD by assessing for exposure to chronic stress, such as bullying. Furthermore, we were underpowered in the present study, and therefore, negative findings should be interpreted with caution, particularly with regards to behavior counts during the TSST and the lack of correlation between behavior and cortisol response in ASD. Due to the small sample size, we were unable to test for potential subtypes within the ASD group who may show a cortisol response to the TSST. Finally, we were not able to assess for sex differences or sex steroid hormone levels and their effect on cortisol reactivity. Additional studies are needed that assess for androgen levels and their relationship to cortisol in adolescents with ASD.

This is the first study to investigate HPA axis and behavioral responses to social evaluative threat in adolescents with ASD. There is a great need for work that centers on the needs of individuals with ASD as they make the difficult transition to adolescence and more complex social relationships. Particularly, longitudinal studies that address the psychosocial and neuroendocrine features of adolescent development in ASD will help to elucidate the mechanisms by which adolescents with ASD can make successful transitions to adulthood and positive social relationships.

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