Biobehavioral Response to Social Judgment in Adolescents with Autism Spectrum Disorder

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Autism Spectrum Disorder: Clinical Characteristics

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impairment in social communication and restricted or repetitive interests or behaviors that are present across multiple contexts and are persistent, beginning in early childhood (APA, 2013). ASD is a broad diagnosis, updated for the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-5), that includes the previously separate diagnoses of autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger Syndrome (APA, 2013). Social communication differences in ASD may include delayed or minimal language acquisition, diminished joint attention (Lewy and Dawson, 1992), difficulties understanding complex or figurative language (Kerbel and Grunwell, 1998), or difficulty taking the perspective of other social agents (Reed, 1994; Travis et al., 2001; Conson et al., 2015). Restricted or repetitive interests or behaviors can include repetitive movements such as hand flapping or rocking, insistence on sameness, routines, or rule-following, or unusual, highly specific interests, such as obsessive focus on a particular video game or movie, or preoccupation with a specific activity or hobby to the detriment of other interests or activities, oftentimes without an interest in a broader context (Bishop et al., 2006; Szatmari et al., 2006; Smith et al., 2009). The use of the term "spectrum" emphasizes the broad range of abilities and challenges that individuals with ASD can have, which may, in addition to the core features in social communication, also include difficulties with sensory hyper- or hyposensitivity (Lord, 1995; Rogers et al., 2003; Baum et al., 2015), and poor muscle tone or delayed motor development (Ming et al., 2007). Increasing evidence also suggests a relative strength for enhanced local visual perceptual processing among many individuals with ASD (Plaisted et al., 1998; Mottron et al., 2003; Almeida et al., 2014). It is important to recognize the variability with the ASD diagnosis across all of these domains. Although many of these features are more prominent in individuals with ASD, they are not

diagnostic, as ASD is defined fundamentally as an impairment of social communication and cognition (Constantino and Charman, 2015).

ASD is highly heritable, with heritability estimates ranging from 30 to over 60 percent (Folstein and Rosen-Sheidley, 2001; Abrahams and Geschwind, 2008; Sandin et al., 2014). The genetics of ASD are extremely complex, and there is debate about whether ASD can be linked to multigene interactions of relatively common functional variants or to aggregate effects of large numbers of *de novo* mutations (Pickles et al., 1995; Veenstra-Vanderweele et al., 2004; Abrahams and Geschwind, 2008). There is also growing evidence for environmental and epigenetic factors that may contribute to the ASD phenotype, including exposure to prenatal stress (for review, see Koufaris and Sismani, 2015), and pro-inflammatory processes in utero (Angelidou et al., 2012), as well as preliminary studies showing a correlation between exposure to pesticides, heavy metals, and phthalates and incidence of ASD (for review, see Kalkbrenner et al., 2014). Prevalence of ASD is increasing, with current estimates at 1 in 68 children, although it is unclear if the increased prevalence is due to a true increase in incidence, better public awareness of ASD and improved access to pediatric care, or the broadening of diagnostic criteria to include more people who would previously not been diagnosed (ADDM, 2014).

A growing body of literature suggests that there may be underlying physiological processes that could impair ability to engage with the social world in ASD (Hutt et al., 1964; Malow et al., 2006; Weisman et al., 2012; Taylor and Corbett, 2014; Tordjman et al., 2015). Because both the hypothalamic pituitary adrenal (HPA) axis and the autonomic nervous system (ANS) mediate approach and avoidance behavior, including social approach and avoidance, these systems are of great interest in the study of ASD. Indeed, increasing evidence points to dysregulation of both the HPA axis and the ANS in ASD, via measurement of cortisol responsiveness (Taylor and Corbett, 2014), and heart rate variability (HRV) measurements following exposure to challenge or stressors (Benevides and Lane, 2015). Given the core importance of social cognition and behavior in the ASD diagnosis, there is a clear impetus to better study the effects of specific social stressors on both the HPA axis and the ANS.

Adolescent Social Development in Autism Spectrum Disorder

There is a developing recognition of the need for research that focuses on adolescence, including research that centers on the goals of individuals with ASD and their families (Carter et al., 2013; Giarelli et al., 2013). There are important changes in the clinical presentation of ASD during adolescence (Schall and McDonough, 2012). By adolescence, the stereotyped behaviors and developmental delays in language acquisition which may have led to first diagnosis are likely no longer present or greatly diminished (Gilchrist, 2001). However, not all symptoms have improved: Parent report data shows limited social engagement with peers for many adolescents with ASD (Shattuck et al., 2011). The primary challenge of adolescence is the preparation for adult social bonds; thus, struggles with social relationships often become more prominent for individuals with ASD as they enter adolescence (Cadman et al., 2012). A recent review has suggested that self-determination, i.e., the ability to "identify strengths, preferences, and interests" and then make choices based on this self-knowledge is a significant struggle for individuals with ASD while making the transition to adulthood (Schall et al., 2012, pp201). Furthermore, self-determination skills are developed during adolescence with increasing responsibility, freedom, and self-competence (Wehmeyer et al., 2013). For individuals with ASD, social skills training and a sense of peer acceptance can facilitate these skills (McGovern and Sigman, 2005; Schall et al., 2012).

Evidence for Alterations in Physiologic Arousal in Autism Spectrum Disorder

ASD is a highly heterogeneous disorder, creating diagnostic and study challenges for clinicians and researchers, respectively. Better characterization of phenotypes within the diagnosis could guide individualized treatment programs. Early work attempting to categorize individuals with ASD on the basis of behavioral phenotypes posited that there may be subtypes within the diagnosis. Specifically, Lorna Wing and Judith Gould proposed a model of three ASD subtypes on the basis of social approach or avoidance behavior (1979). However, the biological correlates of behavioral subtypes in ASD have proven elusive, and to date, the most consistent predictive characteristic of positive outcome following intervention is intellectual ability (McGovern and Sigman, 2005). Despite this, a number of recent studies have identified diverse responses to

social stimuli and their biological correlates in ASD, indicating both hyper- and hypoarousal, as well as behavioral profiles characterized by social approach (of the "active but odd "typology described by Wing and Gould), as well as social avoidance (Rice et al., 2012; Lynch et al., 2013; Schupp et al., 2013; Yager and Iarocci, 2013; Corbett et al., 2014; Roy and Chiat, 2014). There is recognition among clinicians and researchers of the need for enhanced understanding of the neurobiological mechanisms that facilitate arousal in ASD.

This chapter will address the physiological response to challenge and stress in typical development, followed by a discussion of physiological arousal and response to stress in ASD.

The Biological Correlates of Arousal and the Stress Response

Physiologic activation, also termed arousal, underlies a range of behaviors critical to survival. For example, the ability to regulate arousal in response to dynamic environmental stimuli is necessary for successful social integration, including pro-social behavior with conspecifics in affiliative pair, sexual, and maternal-infant bonding (Sachser et al., 1998; Goetz et al., 2010; Tsoory et al., 2012; Noller et al., 2013; Fairhurst et al., 2014). Physiologic regulation is also crucial in the detection of social threat from rivals (Haemisch, 1990; Huhman et al., 1992; Pineda et al., 1994). A large body of research has investigated the neural and physiological correlates of psychological threats, including social threats; these perceived or potential stressors stand in contrast to physical stressors, such as hunger or dehydration, which are a direct threat to bodily systems that facilitate homeostasis (Dickerson and Kemeny, 2004). Social evaluative threat is one form of social or psychological threat and is defined as a threat that represents a danger of poor evaluation of valued aspects of the self by others (Buske-Kirschbaum et al., 1997; Frisch et al., 2015). When physiological arousal in response to such threats extends beyond an organism's capacity for self-regulation, it is termed stress (Rosenfeld et al., 1992; Koolhaas et al., 2011).

The earliest models of the stress response acknowledged phases of physiological arousal. For example, Seyle's General Adaptation Syndrome model posits that the response to threat occurs in several stages, later determined to be mediated by differing biological systems (Seyle, 1975). Response to threat requires the ability to first perceive threat, then mount and regulate the physiological response to threat, produce a behavioral response (i.e., approach or avoidance), and

finally, down-regulate and return to physiologic baseline (Selye, 1975). The mechanisms for these processes are detailed below.

Central Mechanism for Threat Perception: The Autonomic Nervous System

The autonomic nervous system (ANS) is the core biological system responsible for maintenance of homeostasis in response to environmental challenge. The two branches of the ANS, the parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) work in tandem to modulate arousal (Robertson et al., 2012). At the most basic level, perception of threat occurs before conscious awareness and is facilitated by the ANS. Such bottom-up perception of salient stimuli can be characterized as either a defensive response or an orienting response. A defensive response is characterized by reciprocal activation of the SNS and PNS, such that there is heightened SNS activity in response to perceived threat, and a co-occurring decrement in PNS activity (Quigley and Berntson, 1990). This is the classic so-called "fight or flight" response. Conversely, the orienting response is characterized by co-activation of both the PNS and the SNS, resulting in increased attentional resource allocation to salient stimuli. These responses are facilitated by basic brain stem mechanisms and are thought to represent the phylogenetically oldest and most basic central nervous system mechanisms for facilitating arousal and resource allocation (Robertson et al., 2012).

Measuring ANS Function

In humans, ANS activity is typically measured via electrocardiogram, which allows for indirect measure of both PNS and SNS activity via HRV measures such as respiratory sinus arrhythmia (RSA), as well as impedance cardiography measures, respectively. RSA is thought to be a measurement of vagus nerve function and represents the high frequency fluctuations in heart rate that occur with spontaneous breathing (Berntson et al., 1993). The dorsal motor nucleus of the vagus nerve arises from the nucleus ambiguus of the brainstem reticular formation and synapses on the sinoatrial node of the heart, acting to decrease heart rate. Activity of the vagus nerve on the heart serves a homeostatic function to maintain blood pressure that is facilitated by the

baroreceptor reflex. Baroreceptors are mechanoreceptors located in the auricles. Increases in blood pressure cause distention of heart baroreceptors, which fire with increasing frequency. This information is relayed to the nucleus of the tractus solitarius, which stimulates vagal activity, and therefore, the PNS (see Figure 1). In sum, increases in blood pressure lead to an increase in PNS activity, and therefore, a resultant decrement in heart rate— serving the homeostatic function of reducing blood pressure via a negative feedback mechanism (Nosaka et al., 2004).

PARASYMPATHETIC NERVOUS SYSTEM

NTS

Nucleus Ambiguus

Vagus Nerve

M2

β1

Spinal Nerves

NTS

Expiration

Inspiration

Baroreceptor Reflex

← – – Inhibition

← Excitation

SYMPATHETIC NERVOUS SYSTEM
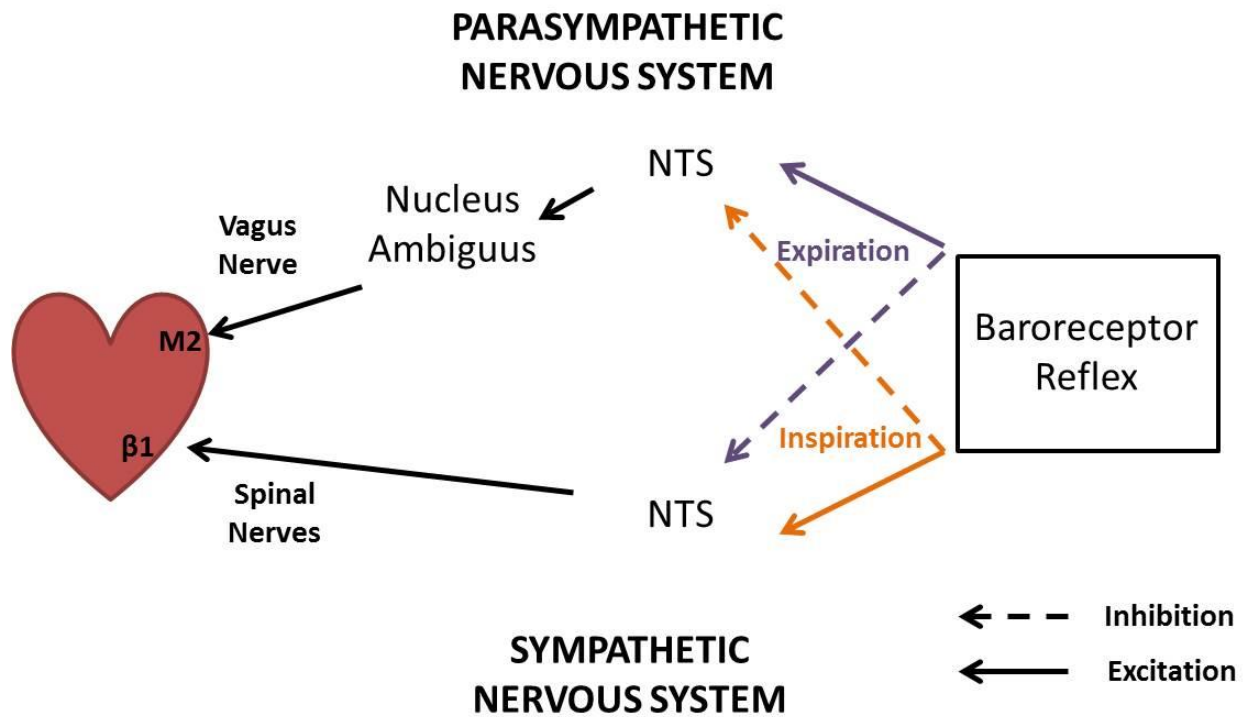
Figure 1. Schematic of ANS effects on heart rate NTS= nucleus tractus solitarius

Although both the PNS and SNS innervate the sinoatrial node, their contributions are separable because of differences in the temporal dynamics of their respective synapses. Because vagus nerve activity is cholinergic and facilitated by the M_2 class of g protein-coupled receptors that facilitate activity of potassium channels, the latency and peak of PNS activity is less than one second. Conversely, the SNS innervates the heart with noradrenergic β_1 receptors that involve a g protein second messenger cascade. The time course of activation for SNS innervation of the

sinoatrial node is therefore much slower, with a latency of about two seconds and a peak of about four seconds (Berntson et al., 1993). Because the peak and latency of PNS innervation happens relatively quickly, it is possible to measure the distinct contributions of vagal innervation of the heart, and therefore, contributions of the PNS to heart rate variability. This so-called "vagal brake" is the core idea of RSA as a measurement of PNS regulatory activity (Porges et al., 1994). Importantly, because the PNS and SNS do not always reciprocally co-activate, alterations in SNS or PNS activity alone cannot be termed a true stress response (Quigley and Berntson, 1990; Gianaros et al., 2001; Berntson et al., 2008). Rather, RSA is conceptualized as an indirect measure of vagus nerve, and therefore, PNS function. Heightened RSA values at baseline are thought to be adaptive, and low RSA values during stressors could indicate physiological response to challenge (Porges, 1992).

In children with ASD, the literature does not suggest group differences in basal or task-based RSA (Benevides and Lane, 2015). Findings of basal RSA are mixed, however, with some studies reporting no group differences (Althaus et al., 1999; Toichi and Makio 2003; Daluwatte et al., 2012; Levine et al., 2012; Watson et al., 2012) and others reporting reduced RSA in ASD at baseline (Ming et al., 2005; Vaughan Van Hecke et al., 2009; Bal et al., 2010). Part of the heterogeneity in the literature is likely driven by the inclusion of pre-pubertal children and adolescences in the same sample (Levine et al., 2012), as well as differences in the definition of baseline and procedures for collecting baseline data. For example, some studies collected baseline while asking participants to watch a movie (Watson et al., 2012), others took baseline with lab staff in the room (Ming et al., 2005; Levine et al., 2012), and others collected baseline data while participants sat alone staring at a blank wall (Althaus et al., 1999; Toichi and Kamio, 2003; Bal et al., 2010). Because of the sensitivity of many individuals with ASD to novelty and environmental sensory stimuli (APA, 2013), these factors are important considerations in the collection of RSA data in ASD.

Although there are not overall differences between ASD and TD groups in the literature, there is evidence for differences in the degree of responsiveness of the PNS to challenge tasks, such that individuals with ASD tend to show reduced change to the stressor than do TD individuals (Althaus et al., 1999; Toichi and Kamio, 2003; Vaughan Van Hecke et al., 2009; Daluwatte et al., 2012). Tasks have included watching a video of a novel adult (Vaughan Van Hecke et al.,

2009), a visual memory challenge (Althaus et al., 1999; 2004), mental arithmetic (Toichi and Kamio, 2003) and sensory exposure challenges (Daluwatte et al., 2012; Schaaf et al., 2015). To date, the only study that has employed a social evaluative threat task and RSA measures did find significantly reduced RSA in ASD compared to TD at all time points, suggesting the value and utility of tasks that involve live actors as social stimuli (Levine et al., 2012).

Promotion and Maintenance of the Stress Response: The Hypothalamic-Pituitary Adrenal Axis

The HPA axis is the primary regulator of the stress response; while the ANS facilitates the initial response to a stressor, the HPA axis amplifies the systemic response to stress, supports regulation of bodily systems, and occurs on a much longer time scale than does ANS activation (Herman and Cullinan, 1997). In the context of threat processing, the paraventricular nucleus (PVN) of the hypothalamus receives excitatory input from the central nucleus of the amygdala, which activates the neuroendocrine cascade, thereby initiating and promoting the stress response (Silverman et al., 1981). Thus, the HPA axis is sometimes termed the limbic-HPA axis, due to the critical involvement of the amygdala in the detection of threat and stimulation of the HPA response (Gunnar and Donzella, 2002). Limbic stimulation of the PVN causes secretion of vasopressin and corticotrophin-releasing hormone (CRH). These peptides act on the anterior pituitary to cause the secretion of adrenocorticotropic hormone (ACTH), which acts on the adrenal cortex. The adrenal cortex produces glucocorticoids (cortisol in humans), which provide negative feedback to the anterior pituitary and hypothalamus by suppressing production of CRH and ACTH (Hennessy and Levine, 1979; see Figure 2). Furthermore, although outside the scope of this review, the sensitivity of each component of the HPA axis is regulated by a number of proteins involved in the trafficking of glucocorticoid receptors (GR), including heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70), thereby controlling GR density and bioavailability (for review, see Pratt et al., 2006; Heitzer et al., 2007). Furthermore, cortisol concentration can impact the binding and concentration of carrier molecules necessary for GR translocation (Nishi and Kawata, 2007). In sum, the regulation of the HPA axis is highly complex and can be influenced by environmental factors such as exposure to chronic or acute stress (Bremne and Vermetten, 2001; Gunnar and Quevedo, 2008; Kertes et al., 2008), as well as

epigenetic mechanisms such as GR transcription regulation (Fish et al., 2004; Meaney and Szyf, 2005), with the end goal being homeostatic regulation of cortisol secretion.
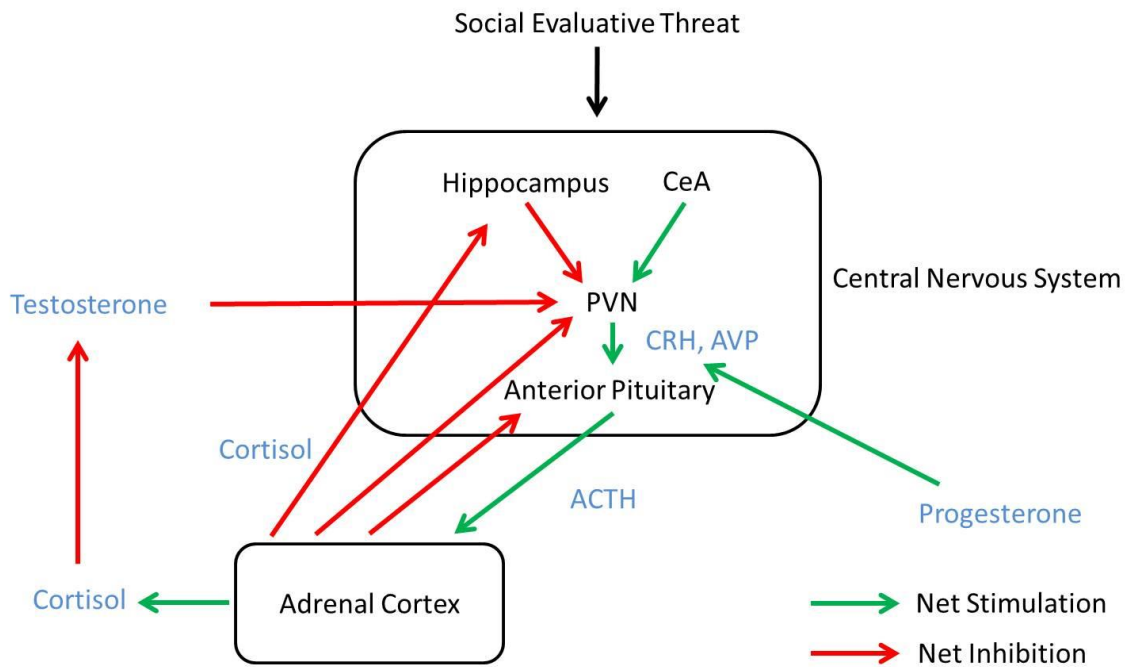
Figure 2. Schematic of HPA axis interactions with limbic and sex steroid systems
PVN=parventricular nucleus of the hypothalamus, CeA=central nucleus of the amygdala, ACTH= adrenocorticotropic hormone

In addition to increases in salivary and plasma cortisol following stress exposure, cortisol is released in a normal circadian pattern. Most individuals exhibit a peak in the morning upon waking, the so-called cortisol awakening response (CAR), followed by a plateau and then a secondary peak in the afternoon, and a decrement in cortisol during the evening (Mason, 1968). Importantly, exposure to stress can change the set-point of the HPA axis, altering basal functioning or overall responsiveness to stress. The effects of stress exposure can be immediate, as is the case with negative feedback loops regulating cortisol production, or long-term, such as epigenetic regulation of receptor transcription. The specific effects of stress exposure on HPA axis function and behavior are largely dependent on the type of stressor and its developmental timing (Sandi and Haller, 2015).

Behavioral Response to Threat and Down-regulation of Arousal

Although the ability to respond quickly to threat is adaptive, prolonged activation of the physiological arousal response is maladaptive and has been linked with immune system dysregulation and psychiatric disorders (Miller et al., 2007). Because of the importance of maintaining homeostasis in response to changing psychological threats, the regulatory processes underlying physiological arousal are complex, finely tuned, and impacted by development. As such, much work has focused on the adaptive role of these regulatory systems, as well as the maladaptive consequences of dysregulated arousal.

The Yerkes-Dodson law states that there is an optimal level of arousal for ideal performance and behavioral response to threat, such that the relationship between arousal and performance functions as an inverse 'u' curve. Furthermore, the optimal level of arousal varies depending on the requirements of the task, and also varies across individuals (Yerkes and Dodson, 1908). More recent research has demonstrated a correlation between glucocorticoid concentration and task performance, measured variably as attention, accuracy, or memory (Lupien et al., 2007). Additional research has shown that threat similarly impacts perceptual abilities (Phelps et al., 2006), and that gains in sensory perceptual abilities are likely caused by increased motivated attention (Lang et al., 1998). One event-related potential (ERP) study in adults with either low- or high-trait anxiety found that during conditions of threat, attention (indexed by the P100 latency) was facilitated in the low anxiety group but not the high anxiety group. The authors posit that for the low anxiety group, threat adaptively impacts biological systems mediating arousal, such that attention is enhanced, whereas, for the high anxiety group, arousal is so heightened that it no longer serves an adaptive function (Laretzaki et al., 2010). Another ERP study of social stimuli salience in the general adult population has shown that the degree of peak reactivity to neutral and threatening faces correlates with both individual differences in rejection sensitivity and social approach behavior, again suggesting that physiological arousal in response to social stimuli is related to both behavior and perhaps even stable, trait-like characteristics (Ehrlich et al., 2015).

Social bonds or inclusion in supportive social groups can serve as a buffer to the stress response (Eisenberger et al., 2007; Giesbrecht et al., 2013). In pre-pubertal children, the maternal presence has a buffering effect on HPA axis activation (Hostinar and Gunnar, 2015), although this effect

is less pronounced with the onset of adolescence (Gunnar and Vazquez, 2001; Hostinar et al., 2015) and is likely mediated by pubertal development (Dahl and Gunnar, 2009; Doom et al., 2015). Peers are less effective at suppressing HPA axis activity in early development (Meyer et al., 1975), but by school age, supportive peer friendships have a demonstrated effect on reducing cortisol response to social evaluative threat and peer rejection (Adams et al., 2011; Peters et al., 2011). Taken together, this literature confirms the critical role of supportive social relationships for the maintenance of homeostasis and health, especially in adolescence.

In the absence of supportive social relationships, there are a number of self-soothing behaviors that appear to be consistent across primate species. These so-called "displacement behaviors" likely evolved from conspecific grooming rituals (Troisi, 2002) and can be defined as any self-directed behavior that serves to distract or disrupt a stressful or challenging context (Mohiyeddini et al., 2013). These behaviors have been associated with a reduction in physiological arousal, as measured by both HRV measures and cortisol response (Pico-Alfonso et al., 2007; Mohiyeddini and Semple, 2013). There have been long-standing hypotheses about the self-soothing effects of idiosyncratic repetitive behaviors such as hand flapping or rocking in ASD (Kinsbourne, 1980). The frequency of such behaviors generally diminishes by adolescence in many, but not all, individuals with ASD (Gilchrist, 2001). The role of more common and socially acceptable displacement behaviors, such as self-grooming or lip biting, has not been studied in ASD.

Developmental Regulation of Physiological Arousal

Developmental effects on basal HRV contribute to the stabilization of ANS function. There is some limited evidence to indicate a stable trajectory of basal HRV, such that fetal HRV is predictive of HRV and psychomotor development at age two (DiPietro et al., 2007). This suggests that basal HRV is a trait-like characteristic that follows a developmental trajectory with a stable slope (Hinnant et al., 2011). A recent review of studies investigating age effects on basal HRV measures in typically developing children found the greatest low frequency to high frequency ratio (LF:HF) in HRV at birth, with a general decrease in LF:HF with age until adulthood. Because PNS activity is thought to be represented by high frequency HRV, the

authors conclude that, overall, there is an increase in PNS activity relative to SNS activity in childhood and adolescence until adulthood (Eyre et al., 2014). The exact cause of age effects on HRV are not known, but adiposity and body size (Dewey et al., 2007) as well as sex steroid hormones (El-Mas and Abdel-Rahman, 2009) may be contributing factors. Several studies of adult women have reported relationships between HRV measures and menstrual cycle phase, with greater vagal activity, as indexed by the HF:LF ratio during the follicular phase, suggesting a relationship between PNS activity and increased estrogen (Sato et al., 1995; Saeki et al., 1997). However, not all studies have found menstrual cycle differences in HRV (Yildirir et al., 2002; Leicht et al., 2003). Importantly, to date, no longitudinal studies of HRV during pubertal transition have been conducted, limiting our ability to draw strong conclusions about developmental effects on ANS function and stability in adolescents.

There are also developmental impacts on HPA axis function. Specifically, the HPA axis undergoes two major periods of development and stabilization that coincide with a surge in gonadal sex steroid hormones: prenatally, and again during the onset of puberty. With regard to prenatal development, bioavailable ACTH can be observed in human fetal pituitary tissue as early as eight weeks (Pavlova et al., 1968). In studies of fetal sheep, a common animal model for human prenatal development, administration of CRF results in increases in ACTH and cortisol by the second trimester (Hargrave and Rose, 1986). Furthermore, amniotic androgen and cortisol levels are correlated in humans, suggesting that the regulation of these hormones is tightly coupled (Sarkar et al., 2007). In pre-pubertal children, HPA axis responsivity is generally more variable than in adults (Elmlinger et al., 2002). As pubertal development progresses, the cortisol response stabilizes, and by late puberty, the cortisol response to stress parallels that of adults, with a fully developed negative feedback response to facilitate down-regulation (Vazquez et al., 1993; Romeo et al., 2004). Converging evidence also points to sex differences in HPA axis responsivity that are likely facilitated by non-estrogen hormones, particularly progesterone and testosterone (Romeo, 2010a). For example, studies have shown differences in cortisol responsivity to stressors in women during differing stages of the menstrual cycle, such that there is greater cortisol responsivity during the luteal phase, which is associated with increased levels of progesterone (Kirschbaum et al., 1999). Rodent studies that allow for the manipulation of endocrine status have shown facilitation of the cortisol response to stressors that correlates with estradiol and progesterone (Redei et al., 1994; McCormick et al., 2002; 2004), while testosterone

seems to decrease responsivity in males (Handa et al., 1994; McCormick et al., 2002). Interestingly, there is generally a greater hypothalamic drive in males than in females, even though testosterone is negatively correlated with cortisol secretion in males (Roelfsema et al., 1993; Kirschbaum et al., 1999). These effects may be mediated by sex differences in inhibitory cortical feedback mechanisms on the HPA axis (Romeo, 2010a).

The Hypothalamic Pituitary Adrenal Axis in Autism Spectrum Disorder

Overall, there is evidence for dysregulation of the HPA axis in ASD, both in terms of variability in diurnal rhythm, but also in response to stressors (for review, see Taylor and Corbett, 2014). However, the literature regarding diurnal cortisol in children with ASD is quite mixed, and although there seems to be evidence for dysregulation, the exact nature of this dysregulation is unclear. For example, some studies have reported group differences in morning cortisol (Curin et al., 2003; Hamza et al., 2010), but most have reported no differences in cortisol at any time point between TD and ASD groups (Richdale and Prior, 1992; Nir et al., 1995; Marinovic-Curin et al., 2008; Kidd et al., 2012). Some studies of cortisol in children with ASD found no differences in the CAR (Corbett et al., 2008; Zinke et al., 2010; Corbett and Schupp, 2014), but significant differences compared to TD children in evening cortisol levels, as well as the rate of decline from peak morning levels to evening levels (Tomarken et al., 2015). It is possible that some of the differences in this literature can be explained by increased inter- and intra-individual variability in diurnal cortisol in ASD (Corbett et al., 2008; Schupp et al., 2013). There may also be differences in the developmental trajectory of the HPA axis in ASD that have not been addressed by the present literature, which is largely cross-sectional and mostly includes pre-pubertal children (Corbett and Simon, 2014).

With regard to acute social stressors or challenge, the most common task in the cortisol literature is the Trier Social Stress Test (TSST) (Buske-Kirschbaum et al., 1997). The TSST is designed to measure the physiological response to social evaluative threat in a controlled laboratory setting. All TSST studies of children with ASD have reported either no increased cortisol response to social evaluative threat (Jansen et al., 2000; Corbett et al., 2012; Lanni et al., 2012, Levine et al., 2012) or reduced cortisol response compared to TD controls (Jansen et al., 2003). These studies

suggest that social evaluative threat is not a salient stressor to children with ASD. However, there has not been a study to date that assesses HPA axis responsiveness to social evaluative threat in a sample of just pubertal adolescents.

Conclusion

Adolescents with ASD are understudied, yet the primary aim of adolescence— social integration and formation of social bonds outside of the family— are particularly challenging for individuals with ASD (Allen and Miga, 2010). Because ASD is defined by impairment in social cognition, studies that address the response to social threat are of critical importance. Furthermore, there is clear evidence for developmental effects on the physiological systems that mediate arousal and the stress response in typical development, including effects of sex steroid hormones associated with the onset of puberty. Given the preliminary evidence for differences in HPA axis and ANS function in pre-pubertal children with ASD, there is a need for research that addresses physiological response to social evaluative threat in adolescents with ASD.

CHAPTER II

SALIVARY CORTISOL AND BEHAVIORAL RESPONSE TO SOCIAL JUDGMENT IN ADOLESCENTS WITH AUTISM SPECTRUM DISORDER

Introduction

ASD is characterized by alterations in social behavior, including impairment in social communication and diminished social insight (APA, 2013). However, it is unclear if the alterations in social behavior in ASD are due to disinterest in social stimuli or heightened anxious arousal in response to perceived or potential social judgment. Because many individuals with ASD experience difficulty identifying or naming their own emotional states (Downs and Smith, 2004; Hill et al., 2004; Griffin et al., 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 but not current-state anxiety (Simon and Corbett, 2013) and trait irritability (Mikita et al., 2015) 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 et al., 2015).

Cortisol is a neuroendocrine hormone that is the principle output of the HPA axis, the primary biologic facilitator of the stress response (Hennessy and Levine, 1979). The HPA axis functions to facilitate adaptive responses to environmental stimuli, including social stimuli (Sapolsky et al., 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 measure the physiologic response to specific social contexts at an approximate 20-minute lag time (Kirschbaum and Hellhammer, 1999).

The majority of studies to investigate salivary cortisol response to social stressors have employed the TSST (Buske-Kirschbaum et al., 1997; 2003). The TSST is a social judgment behavioral

paradigm that requires participants to give a brief speech about themselves in front of an unsupportive audience and is known to elicit a salivary cortisol response in TD children (Buske-Kirschbaum et al., 1997), as well as adolescents (Krishnaveni et al., 2014) and adults (Kirschbaum et al., 1999; Kudielka et al., 2004). Previous studies employing the TSST in children with ASD have suggested that there is a diminished cortisol response to social judgment compared to TD children (Jansen et al., 2000; Lanni et al., 2012; Levine et al., 2012). Some studies have also found significant variability within children with ASD, suggesting that there may be subtypes within the diagnosis who respond to social judgment differently (Jansen et al., 2003; Hollocks et al., 2014), these differences may be due 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 judgment, 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 judgment in ASD. The majority of studies using the TSST in ASD have been with children; however, there has been one study of adults with ASD. 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 stress response to social judgment (Jansen et al., 2006). This indicates that there may be a developmental process by which social judgment becomes salient and stressful to individuals with ASD. To date, no studies have examined the relationship between salivary cortisol and social evaluative threat or social judgment in pubertal adolescents with ASD. The adolescent time period is of particular interest in the study of social judgment, 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 et al., 1986; Roisman et al., 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, 2010b). In TD populations, there are alterations of HPA axis response during puberty that primarily impact the stress response, not basal function (Di Luigi et al., 2006). There is a complex interplay between sex steroid hormones, which become elevated with pubertal onset, and the HPA axis (Payne et al., 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, and therefore increases the amount of biologically active glucocorticoids (McCormick et al., 2002). As such, the rodent literature suggests an increase in the cortisol response to acute stressors in male adolescents compared to male adults (Goldman et al., 1973; Vazquez and Akil, 1993; Romeo et al., 2004; Romeo et al., 2006). Studies in adult human males have also found an increase in testosterone levels that correlates with the cortisol response to the TSST (Lennartsson et al., 2012; Bedgood et al., 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. 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 et al., 1997; Lombardo et al., 2007), and amygdala (Baron-Cohen et al., 2000; Schumann et al., 2009; Groen et al., 2010; Edmiston et al., 2015), share reciprocal connections with the HPA axis (Cullinan et al., 1996; Herman et al., 2003), and are known to undergo significant development during puberty (Giedd et al., 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 prosocial behaviors (Schupp et al., 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 young children with ASD that correlates with physiologic arousal (Ostfeld-Etzion et al., 2015). In studies of play behavior in children, individuals with ASD that engaged in more social behavior demonstrated a heightened cortisol response relative to those who engaged in less social behavior (Schupp et al.,

2013). In additional to prosocial behaviors, behavioral observation techniques can 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, called 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 relevancy to a context, but instead serve as distractors from stressful situations (Mohiyeddini et al., 2013). Displacement behaviors are thought to be adaptive and serve as self-soothing or coping strategies that attenuate the stress response (Troisi, 2002; Mohiyeddini and 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 and Foy, 1987; Levine and 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 judgment, no studies to date have operationalized or investigated behavior during the TSST. Furthermore, there has been no investigation of the relationship between physiological 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 judgment in adolescents with ASD, as well as the behavioral correlates of stress and between-group differences in behavior during the TSST. We hypothesized that 1) ASD adolescents would show a blunted salivary cortisol response to the TSST and 2) 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 3) significant differences between the TD and ASD groups in the rate of displacement behaviors observed during the TSST and that 4) variability in salivary cortisol during the TSST would correlate with the incidence of displacement behaviors during the TSST.

Methods

Participants

Participants included 24 males with ASD and 15 age-matched male participants with TD (for demographics, see Table 1). The Vanderbilt University Institutional Review Board approved this study. Informed consent was obtained from parents prior to participation in the study 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 listserv resources.

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)**	74.88 (11.02)**	36.65 (5.99)*	32.55 (6.31)

Table 1: Participant Demographics 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 $**p<0.0001$ $*p<.05$

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 parent and participant instruction in home saliva sampling methods.

Wechsler Abbreviated Scale of Intelligence (WASI, Wechsler, 1999). All participants completed the WASI and were required to have a Full-Scale IQ of at least 70.

The Pubertal Development Scale (PDS, Petersen, 1988). The PDS is a parent report measure of the degree of physical development including growth in height, growth of body hair, skin changes/acne, and overall development compared to peers. It also includes voice deepening and facial hair questions for males. 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 II (ADOS-II, Lord et al., 2012). Adolescents with ASD completed the ADOS-II, Schedule 3 or 4, depending on developmental appropriateness, to confirm the presence of ASD. The ADOS was administered by research-reliable clinicians (BAC or CN). In the instance of first-time diagnoses, the **Autism Diagnostic Interview-Revised** (ADI-R, Lord et al., 1994) was also administered to a parent. All ASD participants met ADOS-II criteria for ASD with a total ADOS score greater than 7. Two participants who had ADOS-II 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 (BAC, CN).

The Social Communication Questionnaire (SCQ). The SCQ (Rutter, 2003), is a brief parent-report questionnaire that assesses for past and present behaviors indicative of ASD, including social communication abilities and restricted and repetitive behaviors or interests. In the present 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 had a SCQ score greater than or equal to 10 on the SCQ.

The Social Responsiveness Scale Second Edition (SRS-2). The SRS-2 (Constantino, 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. A total t-score was calculated for each participant. 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 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 et al., 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 CST, 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 testing 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 in the lab. Lab personnel took the participant to the preparation room. Following five minutes of preparation, the participant returned to the TSST testing room, where they spoke for five minutes about their qualifications for the fictional lab position. Raters did not provide supportive verbal or nonverbal feedback and maintained a neutral expression throughout. After five minutes, the participant was told to perform five minutes of serial subtraction aloud for the raters. At the end of this task, the raters debriefed the participant, breaking character to smile, provide supportive feedback, and assure the participant that the task was "just pretend." The entire TSST protocol was videotaped by a camera placed on a tripod behind the two raters to allow for systematic analysis of behaviors indicative of a stress response. 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 paradigm's end (see Figure 3). In total, six samples were taken on the day of the TSST.
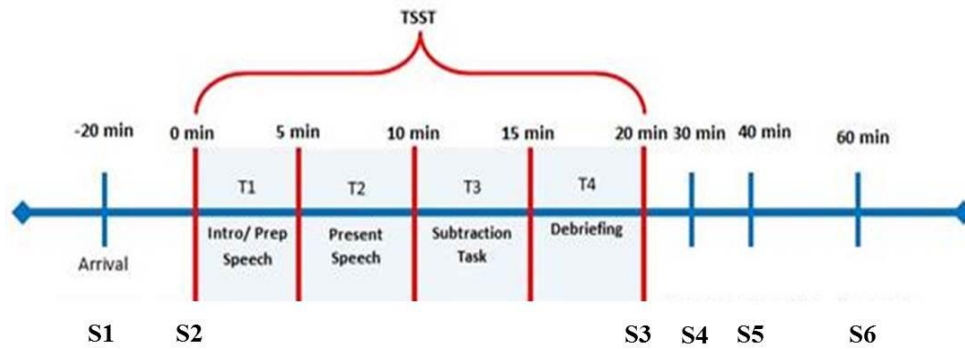

Figure 3. Schematic of TSST and salivary cortisol sampling procedure S1=Sample 1.

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 and the trait form measures anxiety as a stable personality characteristic.

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 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. Frequencies of all of these behaviors were summed for a "Total Stress Behaviors" count. (For a more detailed, operationalized description of behaviors, see Table 2).

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: Operationalized stress behaviors

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 minutes 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 hours, 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 minutes, 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 afternoon basal salivary cortisol levels and state and trait versions of the STAIC.

Cortisol: Descriptive statistics showed positive skewness in the salivary cortisol data. All data was log transformed (base e) to align with the relative normality assumptions of repeated measures ANOVA (Richdale and Prior, 1992; Corbett et al., 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 between-group effects were considered significant at $p < 0.05$.

Repeated measures ANCOVA was used to analyze diagnostic effects on salivary cortisol during the TSST with the salivary cortisol values at each of the six time points on the day of the TSST as repeated measures. Average 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 $p < 0.05$.

Finally, a univariate ANOVA analysis was performed from calculated area under the curve (AUC) data to test for diagnostic differences in total cortisol output. AUC was calculated according to the method described by Pruessner and colleagues (2003), and is a measure of total salivary cortisol output as opposed to peak cortisol output. AUC analysis was performed on the cortisol data collected on the day of the TSST, with average afternoon basal salivary cortisol from the three days of home sampling included as a covariate of no interest. 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 Displacement Behaviors, Fidgeting, and Smiling. *Post hoc* 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

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 1).

Levene's Test demonstrated 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 minutes post-arrival (F=1.668, p=0.205).

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=3.004, p=0.010) but not the ASD group (t=1.620, p=0.119, see Figure 4).
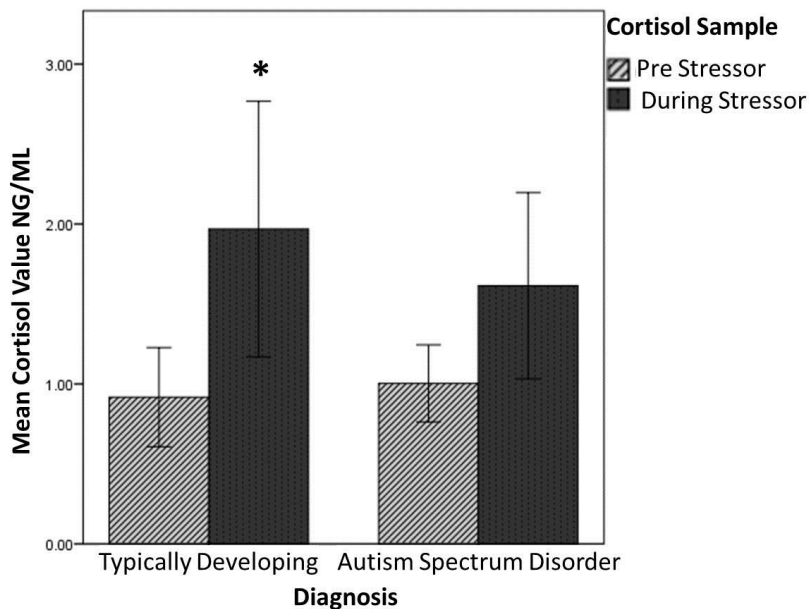

Figure 4: Within-group cortisol response to the TSST

*p<0.05. Pre-stressor value is taken immediately before the onset of the stress paradigm; during stressor represents stress response during the speech and is taken 10 minutes after the stress

paradigm. Raw cortisol values depicted for ease of interpretation; all statistical analyses performed on log-transformed data. Error bars represent SE.

Repeated measures ANOVA revealed no significant differences between groups in salivary cortisol across all six samples when controlling for basal cortisol (F=0.016, p=0.901, see Figure 5). Univariate ANOVA also did not demonstrate any significant between-group differences in AUC (F=0.308, p=.583).
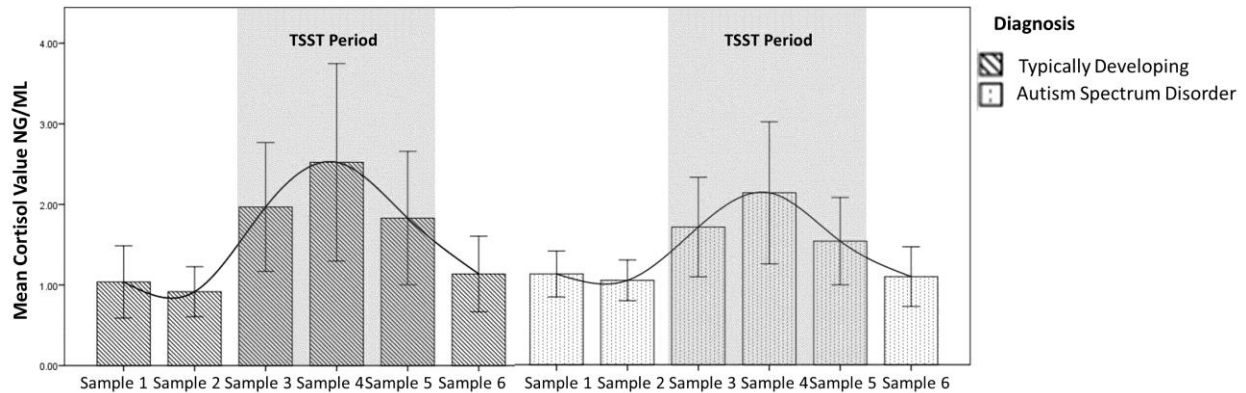

Figure 5: Salivary cortisol during the TSST in ASD and TD adolescents

Unadjusted mean salivary cortisol response across six samples. TSST= Trier Social Stress Test. Raw cortisol values depicted for ease of interpretation; all statistical analyses performed on log-transformed data. Error bars represent SE.

Descriptive statistics revealed extremely low counts of 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 Figure 6).

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. *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 Figure 6).
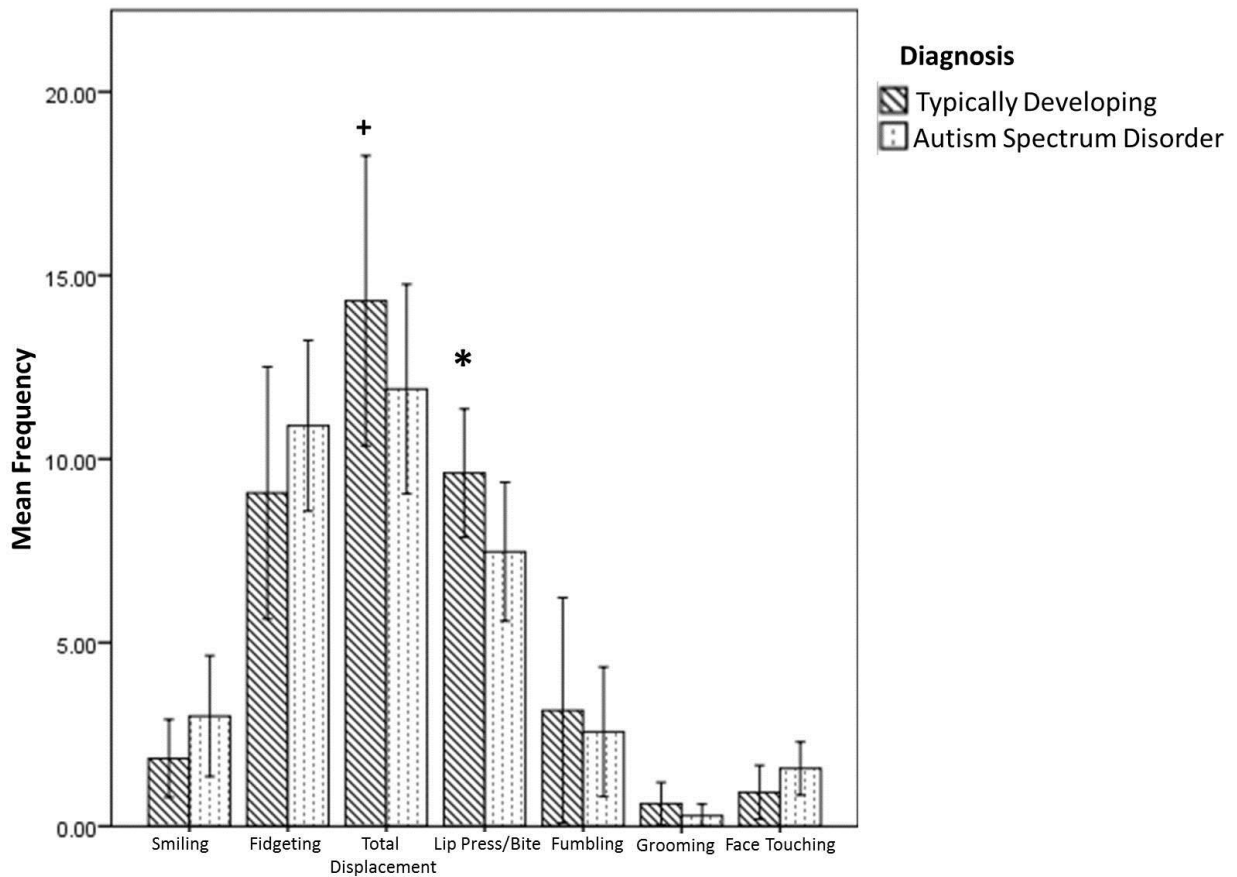
Figure 6: Between-group behavior frequencies during the speech portion of the TSST

+p=0.058, *p<0.05. Error bars represent SE.

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 = -0.552, p=0.050), but not the ASD group (r = -.053, p=0.820, see Figure 7).
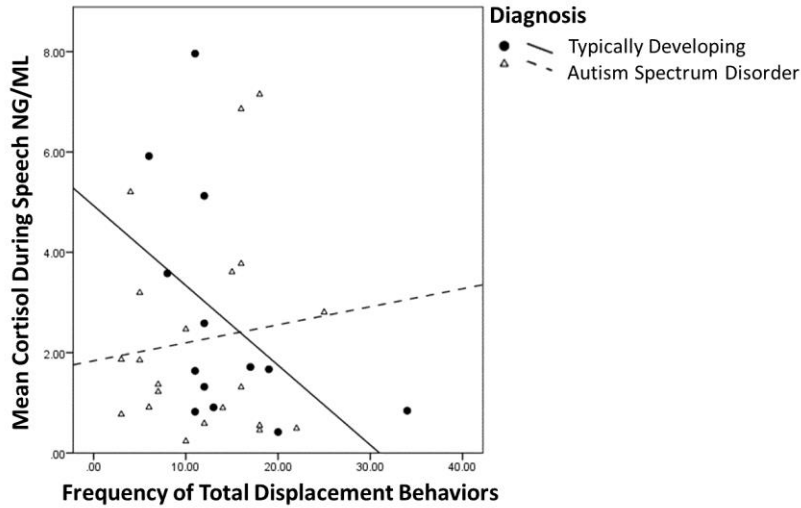
Figure 7: Correlation between Displacement Behaviors and salivary cortisol during the speech portion of the TSST

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 Figure 8).

Figure 8: Correlation between baseline RSA and salivary cortisol measures

Discussion

In this study, we aimed to determine the effects of social judgment on HPA axis responsivity and behavior in adolescents with ASD. As expected, within-group comparison found that for the TD group, the onset of the TSST was associated with 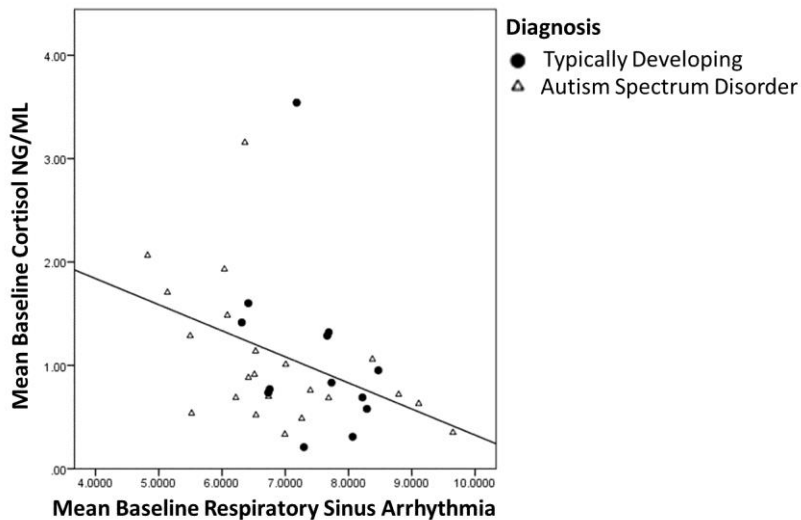a significant increase in cortisol. This increase in cortisol was nonsignificant in the ASD group. We did not find any between-group differences in cortisol response overall between groups.

There was also a negative correlation between baseline cortisol on the day of the TSST and baseline RSA in both the ASD and TD groups, indicating, as expected, that high basal PNS function is associated with reduced HPA axis output. To the best of 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 adults (Cărnuță et al., 2015), but not studies of TD adolescents, which have not found a significant relationship between RSA and cortisol (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.

The findings of the present study substantiate previous work that has found 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 and colleagues (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 judgment 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 coincide with the findings of this study. Given the high rates of anxiety within ASD (van Steensel et al., 2011), the reduced cortisol response to social judgment in the present study could be caused by hypocortisolemia secondary to chronic stress exposure or anxiety; prolonged chronic stress is known to attenuate cortisol production in adults (Martí and Armario, 1997; McEwen, 2004). However, increasing evidence from the 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 adolescents, or for that matter, adolescents with ASD.

Peer bullying and victimization are common challenges for ASD adolescents (Schroeder et al., 2014; De la Iglesia and Olivar, 2015; Fisher and Taylor, 2015; Weiss et al., 2015). It is possible that as individuals with ASD enter adolescence, they develop more insight into their experiences with social marginalization and bullying, although there is a great deal of individual variability in the amount of insight into social victimization that is mediated by theory of mind ability (van Roekel et al., 2010). 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 lesbian, gay, and bisexual young adults, who are extremely likely to experience peer bullying and social marginalization (Burton et al., 2014; Juster et al., 2015). This literature suggests that social support, particularly familial support, can impact the HPA axis response to social stressors, especially during childhood and adolescence in groups that experience social exclusion or bullying (Hostinar et al., 2015). A recent study that altered the degree of status and acceptance threat during a modified TSST in healthy young adults found a relationship between greater degree of threat to social acceptance and a heightened cortisol response. Specifically, in this study, youth were randomly assigned to different versions of the TSST, one "low threat" version which told them their responses would not be evaluated, one "high social acceptance threat" version, where participants were told they would be rated on the basis of their overall likeability, one "high status threat" version where they were told they would be rated on their competency, and finally, a combined version that included instructions from both the social acceptance and status conditions. The authors found the greatest increases in cortisol response to the social acceptance threat, and additive increases for the combined condition compared to the low threat version of the TSST (Smith and Jordan, 2015). Taken together, this research suggests that awareness of social status, or concerns about social marginalization or inclusion, can independently impact the salivary cortisol response to the TSST. Future studies in ASD and other developmental disabilities should assess for the impact of social stigma on individuals' perception and physiological response to peer social judgment, perhaps by assessing for level of insight into peer bullying or other forms of exclusion related to stigmatized identities.

Conversely, it is possible that for individuals with ASD, social judgment in a structured setting such as the TSST is not interpreted as stressful. This may be due to impaired social awareness

and social threat detection in children with ASD (Lanni et al., 2012). One study of adult males with high alexithymia, a poor ability to identify emotions, used the TSST to measure cortisol response to social judgment and found an increased basal cortisol level that was correlated with self-reported difficulty in describing feelings, but not with peak cortisol levels, which did not differ from those of low alexithymia participants (de Timary et al., 2008). This suggests that there may be some relationship between cortisol responsiveness and social insight. Likewise, 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 and Rutherford, 2009). Furthermore, 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 in our lab 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; Corbett et al., 2012; Schupp et al., 2013; Corbett et al.; 2014) and other studies have found a heightened response to play with an unfamiliar peer in ASD (Lopata et al., 2008) and to the strange situation task in toddlers with ASD (Naber et al., 2007). Future studies in ASD populations should assess for degree of social insight, which varies greatly among individuals with ASD, to ascertain if low social insight is responsible for the blunted stress response to social judgment.

With regard to behavior, there was a greater number of Displacement Behaviors in the TD group that was largely driven by the greater frequency of the Lip Press or Bite Behavior. There was also a significant correlation between Displacement behavior counts and salivary 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. This relationship between Displacement behaviors and salivary cortisol response during the speech portion was not present in the ASD adolescents. These findings, although preliminary, may suggest that for TD adolescents, displacement behaviors serve as a self-soothing strategy during times of stress, and are therefore adaptive. Based on the present data, this does not appear to be true for adolescents with ASD.

This study is not without limitations. 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 significantly more trait anxiety than did the TD group post-stressor. However, parental reports of anxiety symptoms did not suggest an effect on cortisol response or behavior. 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 group differences in cortisol responsivity, future studies should examine potential mechanisms for the blunted stress response to social judgment in ASD by assessing for exposure to chronic stress and bullying and individual differences in degree of insight into social judgment. We were not able to include individuals with ASD and intellectual disability due to the cognitive nature of the TSST. Future studies should employ social stressors more suitable for individuals with intellectual disability. Finally, we were not able to assess for sex steroid hormone levels and their effect on cortisol reactivity. Additional studies are needed that assess for androgen levels and their relationship to the cortisol response to the TSST in both male and female adolescents with ASD.

This is the first study to investigate HPA axis and behavioral responses to social judgment 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.

CHAPTER III

RESPIRATORY SINUS ARRHYTHMIA AND SOCIAL JUDGMENT IN ADOLESCENTS
WITH AUTISM SPECTRUM DISORDER

Introduction

The ANS is a core biological regulatory system; it is thought to underlie a host of approach and avoidance behaviors that drive social interaction (Porges and Furman, 2011). ASD is a neurodevelopmental disorder characterized by a primary deficit in social behavior (APA, 2013). Early theoretical models have suggested that a dysregulated ANS may underlie social approach behavior alterations in ASD (Hutt et al., 1964). Because ASD is associated with a wide range of symptoms and co-occuring conditions that impact a host of biological systems (Mazzone et al., 2012), the ANS is a likely candidate for understanding aspects of the biological underpinnings of ASD.

Both the parasympathetic and sympathetic branches of the ANS project to the sinoatrial node of the heart, and are responsible for variations in heart rate. One measure of ANS function is respiratory sinus arrhythmia (RSA), which is a metric of high-frequency heart rate variability (HRV) that occurs with spontaneous breathing, and is thought to measure parasympathetic nervous system (PNS) or "rest and digest" function via cholinergic vagus nerve projections to the heart (Porges, 1992). High baseline RSA values are thought to be related to adaptive social functioning, with studies in TD children indicating that high baseline RSA is associated with less social inhibition and greater empathic responsiveness (Diamond et al., 2011). In contrast, low RSA values have been associated with psychosocial stress (Pico-Alfonso et al., 2007) and effortful emotional regulation during exposure to negative or upsetting video content (Butler et al., 2006). Overall, in TD populations, high RSA is thought to represent the ability to engage flexibly and adaptively with the social world (Porges, 1992), though such abilities are often challenging for individuals with ASD.

There is an emerging ASD literature regarding ANS effects on cardiac function in pre-pubertal children; althought these studies have been somewhat mixed (for review, see Benevides and Lane, 2015). This may be due in part to differences in the type of stressor or challenge used. For example, studies of school-age children with ASD using cognitive or sensory processing tasks have found reduced RSA. In one such study employing a visual search task, the authors found blunted high-frequency HRV in the ASD group compared to TD control children (Daluwatte et al., 2012). Another study of vagal tone in children with pervasive developmental disorder found reduced parasympathetic responsivity during a visual search task with a high attentional load (Althaus et al., 2004). One study that investigated RSA changes in response to sensory challenge found blunted responsivity in ASD children compared to TD children (Schaaf et al., 2015). Studies that employ facial recognition tasks have been more mixed, with two studies reporting no group differences in RSA (Bal et al., 2010; Watson et al., 2012) and one study reporting blunted RSA responsivity (Vaughan Van Hecke et al., 2009). Taken together, the literature in pre-pubertal children with ASD suggest blunted PNS response, but adolescents with ASD are understudied.

In typical development, the adolescent period is critical for the development of key brain regions subserving social functions, including cortical regions linked to the processing of social information, social judgment, and emotional regulation (Eiland and Romeo, 2013), as well as the development and stabilization of neuroendocrine feedback loops that regulate the stress response (McCormick and Mathews, 2010). RSA may serve as a downstream marker of regulatory ability related to cortical development or dysfunction (Beauchaine, 2015). Evidence from TD populations implicates developmental effects on ANS measures such as RSA that occur during puberty (Tanaka et al., 2000; Kowalewski et al., 2007; Jarrin et al., 2015) and correlate with age, with the greatest effects in the adolescent epoch beginning at the onset of puberty (Shahrestani et al., 2015). RSA studies in TD populations suggest ANS stabilization that occurs between the ages of four and five, and then again during adolescence with the onset of puberty (Woodall and Matthews, 1993; Pitzalis et al., 2004), followed by a gradual decline with aging (Jennings and Mack, 1984; De Meersman, 1993). These studies in TD populations suggest that characterization of a pubertal sample of ASD adolescents is warranted.

Adolescence is a time of increasing social complexity, wherein peers take on more importance and salience relative to adults (Brown et al., 1986; Roisman et al., 2004). Adolescence is also linked to increased stress, in part due to peer bullying and social judgment (Espelage and Holt, 2001), but also due to the increasing external and environmental demands that are placed on individuals as they begin the transition to adulthood (Compas et al., 1995). This period is also linked to the onset of internalizing problems such as anxiety (Hayward, 2003). Anxiety can be operationalized as a state of heightened stress or physiologic arousal during anticipation of a real or perceived potential threat; anxiety disorders occur when chronic anxiety interferes with day-to-day functioning (APA, 2013). An emerging literature suggests high rates of co-occuring internalizing disorders, including anxiety disorders, throughout the lifespan in ASD (Matson and Nebel-Schwalm, 2007; Charlot et al., 2008; Gjevik et al., 2011), as well as increased anxiety symptoms (Muris et al., 1998). For example, many individuals with ASD demonstrate an altered physiological stress response to social stimuli; some children with ASD have a heightened response and others a blunted response to social stimuli (Corbett et al., 2012). Several studies have demonstrated correlations between RSA and anxiety symptoms in ASD (Moskowitz et al., 2013; Guy et al., 2014; Kushki et al., 2014), indicating that co-occuring conditions may play a prominent role in physiologic variability within ASD. The increasing nuance needed to understand and navigate the social world with the onset of adolescence likely creates a significant challenge to ASD adolescents. Surprisingly, little work has been done to investigate the psychophysiological correlates of social stress in the adolescent period, despite the fact that co-occuring problems such as anxiety may have significant impact on functional abilities and quality of life for youth with ASD (Dubin et al., 2015; Pellecchia et al., 2015).

Thus, there is a great need for research that aims to understand the role of social stress and anxiety in ASD, particularly research that investigates the underlying physiology that impacts social impairment. A few studies have used computerized social tasks such as facial affect recognition (Bal et al., 2010) or videos of familiar and unfamiliar adults (Vaughan Van Hecke et al., 2009; Watson et al., 2012) and found either no between-group differences in RSA (Bal et al., 2010; Watson et al., 2012) or blunted RSA (Vaughan Van Hecke et al., 2009).

There have been almost no RSA studies that use naturalistic social situations that include real-time social actors. One such study measured RSA during completion of a 10-minute play-based

observational assessment of social behavior with an adult; in this study, baseline RSA in children with ASD was correlated with frequency of social behavior during the observational assessment of social behavior. Specifically, higher baseline RSA was associated with more conventional gestures and sharing behavior with the adult social actor (Patriquin et al., 2013). However, no studies to date have used peer actors in naturalistic settings to investigate RSA reactivity in adolescents with ASD.

Measuring the response to social stress is often conducted by examining a known trigger of the stress response, social evaluative threat, or social judgment (Mason, 1968). One classic experimental paradigm used to study social evaluative threat is the Trier Social Stress Test (TSST), first developed for use in adults (Kirschbaum et al., 1993) and later adapted for use in children (TSST-C) (Buske-Kirschbaum et al., 1997). Briefly, participants are given five minutes to prepare a speech in front of two unsupportive adult raters. They then have five minutes to deliver the speech, followed by five minutes of serial subtraction and a debriefing period. In child and adult populations without neurodevelopmental disorders, the TSST has demonstrated reliable increases in the stress response using both neuroendocrine (for review, see Foley and Kirschbaum, 2010) and psychophysiological markers of stress responsivity (for review, see Allen et al., 2014). When the TSST has been implemented in neural-cardiac studies of children with ASD, findings have been mixed; two studies have reported reduced responsivity in ASD (Hollocks et al., 2014; Mikita et al., 2015) and one study found no differences (Levine et al., 2012) from TD controls. This inconsistency in findings is likely due to methodological differences and study design, including differences in the type of physiological marker measured, as some studies have focused on group differences in RSA and others on within-group heart rate changes between baseline and the task. The RSA literature in ASD is likely complicated not only by differences in task design, but also by heterogeneity in ASD samples, both within and across studies. Recent expansion of the diagnostic criteria for ASD has contributed to this heterogeneity and underscores the importance of a carefully characterized clinical sample (APA, 2013). This characterization includes co-occurring psychiatric conditions common in ASD, such as anxiety disorders (van Steensel et al., 2011), which may further complicate and confound investigations of ANS function. The contribution of anxiety symptoms to the stress response in ASD is critical, as some individuals with ASD may be indifferent to social judgment, whereas others may be hypersensitive to it (Corbett et al., 2012).

The purpose of this study is to examine social stress in adolescents with ASD. In order to measure physiologic arousal and regulatory capacity in response to social stress, we measured PNS function using RSA. RSA can be measured in a minimally invasive way, via electrocardiogram, and therefore allows for mobile measurement of neural-cardiac data in real time. In order to invoke social stress, we employed the TSST, which requires participants to actively engage and interact flexibly with novel social actors in real time. To date, there have been no studies of pubertal adolescents with ASD that have measured RSA during the TSST. We adapted the TSST for use in an adolescent sample by replacing one of the adult raters with an age-matched peer adolescent to provide more social salience to the task. Previous study has shown enhanced responsivity to peers in ASD, particularly novel peers (Corbett et al., 2013), which is in line with the increased salience of peers during this developmental period. We hypothesized that 1) adolescents with ASD would show a blunted stress response to the TSST compared to TD adolescents, but that 2) there would be significantly more variability in RSA responsivity within the ASD group compared to the TD group, and that 3) this variability would be correlated with social and anxiety symptom profiles in ASD.

Methods

Participant Recruitment

TD participants were recruited by word-of-mouth, from university-wide email announcements, community events, and fliers. Participants with ASD were recruited via word-of-mouth and referral from university-based clinical practices, area schools, community advocacy group mailers, community events, and healthcare providers. All subjects were recruited and consented within accordance of ethical standards set forth by Vanderbilt University Institutional Review Board Human Subjects protocols. All procedures performed in this study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Medical Inclusion Criteria

Twenty-five ASD (4 female) and 17 TD (4 female) adolescents between the ages of 12 and 18 years participated in this study (see Table 3a for demographic information). All TD participants had no current or past history of psychiatric or developmental disability or current psychotropic medication usage. ASD participants were not currently taking any antipsychotics or steroid medications and those prescribed stimulants abstained from usage immediately prior to and on the day of the behavioral paradigm, as these substances are known to interfere with the physiologic stress response (Granger et al., 2009).

Group (N)	Age (SD)	PDS Score (SD)	Verbal IQ (SD)	Performance IQ (SD)	FSIQ (SD)	SCQ Total Score (SD)	SRS Total T Score (SD)
TD (17)	15.17 (1.69)	3.04 (0.69)	119.12 (13.05)	112.00 (9.56)	117.65 (10.45)	1.82 (2.01)	42.18 (2.98)
ASD (25)	14.93 (1.51)	3.14 (0.49)	101.40 (23.14)**	106.52 (15.86)	104.28 (20.43)*	21.12 (10.60)***	76.24 (10.85)***

Table 3a: Participant demographics, males and females FSIQ=Full Scale IQ, SCQ= Social Communication Questionnaire, SRS=Social Responsiveness Scale *p<0.05; **p<.01; ***p<0.0001

The present study consisted of two lab visits, the first of which involved administration of clinical assessment to confirm diagnosis of ASD and neuropsychological measures. The following forms were completed on the first visit to the laboratory:

Participant Neuropsychological and Clinical Measures

Wechsler Abbreviated Scale of Intelligence (WASI, Wechsler, 1999). All participants completed the WASI and were required to have a Full-Scale IQ of at least 70.

The Pubertal Development Scale (PDS, Petersen, 1988). The PDS is a parent report measure of the degree of physical development including growth in height, growth of body hair, skin changes/acne, and overall development compared to peers. It also includes voice deepening and facial hair questions for males. 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 II (ADOS-II, Lord et al., 2012). Adolescents with ASD completed the ADOS-II, Schedule 3 or 4, depending on developmental appropriateness, to confirm the presence of ASD. The ADOS was administered by research-reliable clinicians (BAC or CN). In the instance of first-time diagnoses, the **Autism Diagnostic Interview-Revised** (ADI-R, Lord et al., 1994) was also administered to a parent. All ASD participants met ADOS-II criteria for ASD with a total ADOS score greater than 7. Two participants who had ADOS-II 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 (BAC, CN).

The Social Communication Questionnaire (SCQ). The SCQ (Rutter, 2003), is a brief parent-report questionnaire that assesses for past and present behaviors indicative of ASD, including social communication abilities and restricted and repetitive behaviors or interests. In the present 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 had a SCQ score greater than or equal to 10 on the SCQ.

The Social Responsiveness Scale Second Edition (SRS-2). The SRS-2 (Constantino, 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. A total t-score was calculated for each participant. 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 t-score greater than 50.

Child Behavior Checklist (CBCL), The CBCL is a parent report of problem behaviors that occur in childhood across multiple domains (Achenbach, 1991). The questionnaire asks parents to rate the frequency of behaviors using a Likert scale ranging from 0="Not True" to 2="Very Often True". Scores are summed to create a t-score for various syndromes. In the present study, we examined the Anxiety Problems subscale, as well as the Internalizing Problems subscale, which is a summed measure of the symptoms reported in the following subscales: Anxious/Depressed, Withdrawn/Depressed, and Somatic Complaints.

The following procedures were completed on the second visit to the laboratory.

Electrocardiogram Apparatus

To calculate respiratory sinus arrhythmia (RSA), participants wore a mobile multichannel BioNex 8-slot chassis electrocardiogram unit that included a 4-channel biopotential amplifier (MindWare Technologies, Grahanna, OH) during the TSST behavioral paradigm visit. Upon arrival for the TSST, participants were escorted to a testing room with lab personnel they had previously encountered during neuropsychological testing. After allowing for 20 minutes to adjust to the new environment, lab personnel introduced the mobile ECG unit with a cartoon depicting electrode placement on the torso. Participants were then shown the electrodes and allowed to "practice" placement on their hand to allow for sensory accommodation to the sensor adhesive and gel. This was done to mitigate potential ANS responses to sensory novelty. Sensors were placed on the participant's torso following standard ECG protocols. Participants were asked to sit quietly for five minutes while baseline ECG data was collected. The participant wore the mobile unit for the remainder of the paradigm.

Trier Social Stress Test Paradigm

Following completion of the baseline ECG, participants began the social stress protocol. The TSST is a psychosocial paradigm known to elicit a physiological stress response in a controlled laboratory setting (Buske-Kirschbaum et al., 1997). The TSST is a 20-minute paradigm consisting of four five-minute components: 1) Preparation Period, 2) Speech Delivery Period, 3) Serial Subtraction Period, and 4) Debriefing Period (see Figure 9).
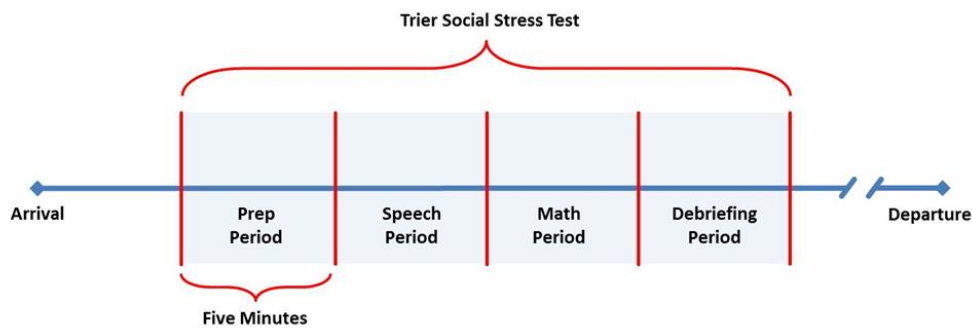

Figure 9: Schematic of Trier Social Stress Test paradigm

Adolescents were escorted to the TSST room, which was a conference room where they were greeted by two raters wearing white lab coats and holding clipboards, sitting behind a table. A video camera was set up behind the raters to film the participants during the paradigm. Both raters were trained not to provide verbal or nonverbal feedback during the TSST and each maintained a neutral affect throughout the protocol. In order to increase the salience of social judgment for the adolescent sample, one of the raters was an age-matched peer. One rater was female and one rater was male. The adult rater asked the participant to stand at a microphone facing the table, and then informed the participant that they had five minutes to prepare a speech about why they would be the best candidate for a job. They would then be asked to deliver this speech before the selection committee of experts and that their speech would be compared to others'.

The participant was then escorted back to the testing room and told to sit quietly for five minutes of preparation. Participants were not permitted to take notes or use aides of any kind, and any questions directed at lab personnel were ignored. At the end of five minutes, the participant was escorted back to the TSST room, where they then had five minutes to deliver their speech. If the participant stopped speaking before the five-minute time period ended, the adult rater prompted them with a series of questions delivered in a neutral tone, such as, "Tell us about your previous job experience," and "Why should we pick you for the job over everyone else?" Following the speech delivery period, the participant was instructed to begin the serial subtraction task, which required them to subtract the number 7 from 758 serially out loud. If the participant made a mathematical error, they were asked to stop and start again from the beginning. If the participant made more than five errors, the rater asked them to subtract by three, starting at 307. After five minutes, the raters begin debriefing the participant by explaining that their performance will not be judged and that the entire task was "just pretend." The raters showed positive affect, praised the participant's performance, and provided support and encouraged the participant to ask questions. The raters then thanked the participant for taking part in the study.

Electrocardiogram Data Processing and Respiratory Sinus Arrhythmia Calculation

RSA was calculated minute by minute from each participant's ECG trace using the BioLab 2.4 Heart Rate Variability Software Suite provided by MindWare Technologies. This software suite allows for automated recognition of R peaks on the ECG trace. All data were further inspected and processed to confirm the presence of a clear R peak and the absence of cardiac abnormalities such as arrhythmias. Data were also checked to ensure respiration rates were within the expected physiological range (.15-.40 Hz). Data outside of this range were not included in the study and accounted for .48% of the total data collected (four minutes).

Of the total collected data, 4.3% had to be discarded due to excessive motion artifact that interfered with the identification of R peaks. All data included for further statistical analysis had a confirmed respiratory frequency within the expected physiological range of .15-.40 Hz, a clear ECG waveform, including an R peak, and spanned at least ten respiratory cycles.

Statistical Analysis Plan

All statistical analyses were performed using SPSS Version 22.0 (IBM Corp, 2013) Independent t-tests were used to examine group differences in demographic and clinical variables (see Tables 3a and 3b). These include pubertal stage, verbal and performance IQ, SRS and SCQ scores.

Levene's Test for Homogeneity of Variance was performed to test for significant between-group differences in RSA data variability. To test for effects of diagnosis on RSA during the TSST, a Repeated Measures ANOVA was conducted with RSA as the dependent variable and pubertal status as a covariate of no interest.

Pearson partial correlation analyses controlling for baseline RSA were performed to assess for symptom correlates of RSA using the Internalizing Symptoms and Anxiety Problems t-score subscales of the CBCL. Pearson bivariate correlations were also performed to assess relationships between baseline RSA and the SRS and SCQ total t-scores.

Results

Preliminary descriptive statistics showed significant differences in variability between males and females, driven largely by the female ASD group (see Appendix). Because we were underpowered to detect significant main effects of diagnosis by sex on RSA, we opted to remove the eight females and proceed with a sample of adolescent males only. The final sample contained 13 TD participants and 21 ASD participants (see Table 3b).

Group (N)	Age (SD)	PDS Score (SD)	Verbal IQ (SD)	Performance IQ (SD)	FSIQ (SD)	SCQ Total Score (SD)	SRS Total T Score (SD)
TD (13)	14.77 (1.70)	2.80 (0.60)	118.15 (14.44)	112.92 (9.33)	117.69 (11.63)	2.23 (2.09)	42.08 (3.12)
ASD (21)	14.99 (1.35)	3.04 (0.46)	104.24 (23.90)	108.86 (15.92)	107.19 (20.93)	21.67 (10.48)***	75.00 (10.92)***

Table 3b: **Final sample demographics, males only** FSIQ=Full Scale IQ, SCQ= Social Communication Questionnaire, SRS=Social Responsiveness Scale *p<0.05; **p<.01; ***p<0.0001

Independent samples t-tests revealed no significant pubertal stage, verbal, or performance IQ between groups. There was a significant difference between groups in SCQ and SRS total scores (see Table 3b).

Levene's Test revealed a significant difference in baseline RSA variability between groups (F=4.174, p=0.049, see Figure 10), with the ASD group showing more variability than the TD group. There were no significant between-group differences in variability during the other portions of the TSST (all p values >0.05). Thus, hypothesis two was partially supported.

Repeated Measures ANOVA demonstrated a significant main effect of group on RSA (F=4.97, p=0.033), with lower RSA values in the ASD group (see Figure 10). There was no group by TSST time period interaction effects. Thus, our primary hypothesis was partially supported (see Figure 10).
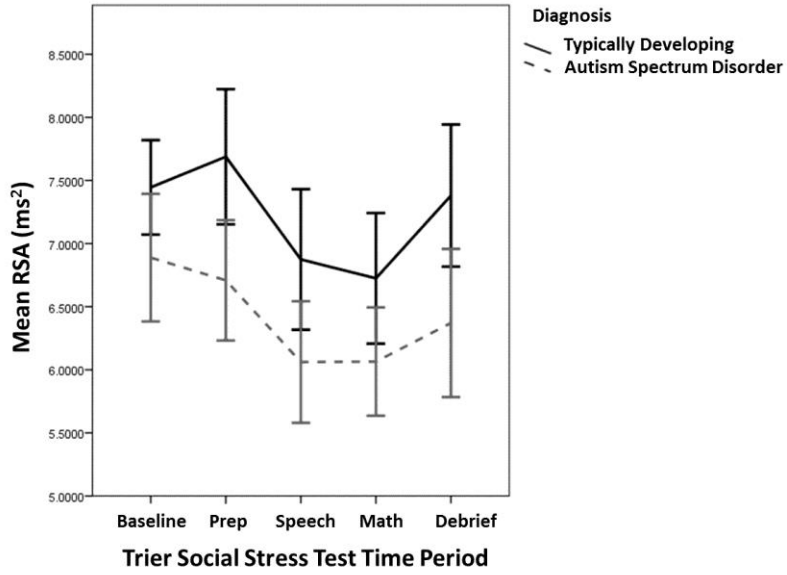
Figure 10: Main effect of diagnosis on Respiratory Sinus Arrhythmia ASD=Autism Spectrum Disorder, TD=Typical Development, RSA=Respiratory Sinus Arrhythmia. Error bars represent SE.

There was a significant negative correlation between baseline RSA and SRS total score (r= -0.346, p=0.045, see Figure 11). There was no significant correlation between SCQ and baseline RSA.

Figure 11: Correlation between baseline RSA and Social Responsiveness Scale total score in Autism Spectrum Disorder

Partial correlations controlling for baseline RSA revealed significant relationships between RSA during the debriefing period and the Anxiety Problems (r=-0.389, p=0.027) and Internalizing Problems (r=-0.385, p=0.027) subscales of the CBCL.

Discussion

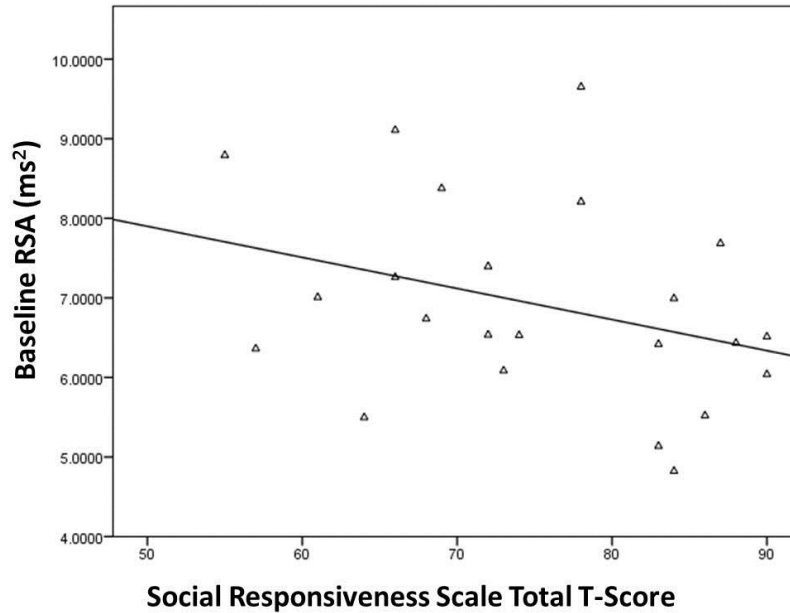In the present study of adolescent males, we aimed to determine the physiologic stress response to social judgment via measurement of RSA. Participants with ASD showed lower RSA values compared to TD males, both at baseline and during the TSST. These findings indicate altered overall physiologic self-regulation in ASD adolescent males. *Post hoc* investigation of relationships between ASD symptoms and RSA values did not reveal correlations during the experience of social judgment itself, but instead, significant relationships at baseline and during the debriefing period following the social judgment stressor. Specifically, RSA was correlated with SRS total t-scores at baseline in the ASD group, suggesting that more severe ASD symptoms are associated with lower PNS function. Furthermore, within the ASD group, parental reports of both internalizing and anxiety symptoms were correlated with RSA following the TSST. These findings suggest that for adolescents with ASD and significant anxiety symptoms, there may be difficulty in "recovering" or returning to baseline following exposure to social stressors, while greater overall ASD symptom severity may be more closely related to physiological functioning at baseline.

 Our findings do not indicate a group responsivity difference between ASD and TD adolescents during the social stressor per se, but rather an overall dampening of PNS function in the ASD group across all time points. These findings suggest that the onset of the social stressor was not a primary between-group differentiator of PNS function, but that the ASD group overall shows a dampened PNS response that was present at baseline and persisted throughout the task and during the recovery period. Our findings confirm and extend previous work highlighting the relationship between baseline RSA and various measures of social ability and prosocial behavior in both clinical and community samples (D'Antono et al., 2005; Hastings et al., 2008; Diamond et al., 2011; Hinnant and El-Sheikh, 2009; Beauchaine et al., 2013; Graziano and Derefinko, 2013; Patriquin et al., 2013; Sulik et al., 2013; Taylor et al., 2015).

In this study, we found correlations between baseline RSA and ASD symptom severity as measured by the SRS, as well as correlations between RSA following exposure to the social stressor and internalizing and anxiety symptoms. Porges' Polyvagal Theory (1995) argues that high baseline RSA denotes greater ability to react flexibly and adaptively to the changing demands of the environment and that high baseline RSA supports adaptive coping in the face of

environmental challenges. Because the SRS measures social awareness and social motivation, it is possible that lower PNS function at baseline broadly impacts ASD individuals' ability to engage with the changing social environment. The literature regarding baseline PNS differences in ASD has been varied, with some reporting no differences between ASD and TD groups at baseline (Althaus et al., 1999; Toichi and Kamio, 2003; Daluwatte et al., 2012; Levine et al; 2012; Watson et al., 2012), and some showing decreased basal PNS function in ASD (Ming et al., 2005; Vaughan Van Hecke et al., 2009; Bal et al. 2010). Consistently, no studies show increases in PNS measures in ASD compared to TD groups. Our findings of a relationship between social symptom severity and baseline RSA, as well as anxiety and internalizing symptom severity and RSA during the debriefing or recovery period following the stressor indicate that perhaps some of the variation in the ASD RSA literature may be due to symptom profile, highlighting the heterogeneity within the ASD diagnosis, including co-occurring anxiety or other internalizing disorder symptoms. It appears that careful clinical characterization is important for determining the relationship between physiologic regulation and social judgment in this population.

To date, there have been only a few studies using the TSST to investigate psychophysiological response to social judgment in ASD. For example, one study by Levine and colleagues (2012) examined vagal tone, which the authors define as beat-to-beat heart rate variability and describe as a measure of PNS function . The Levine study included pre-pubertal children with ASD and employed the TSST-C, a version adapted for children (Buske-Kirschbaum et al., 1997). The authors did not find significant differences in ANS function between TD and ASD groups, but there was a non-significant overall reduction in vagal tone in the ASD group at baseline and during all portions of the TSST-C, which parallels the present findings. Another study used a stress test similar to the TSST, but, instead of a serial subtraction task, asked participants to complete the Rey-Osterrieth Complex Figure task (Mikita et al., 2015). This study measured heart rate as a proxy for stress reactivity to the task, but did not measure HRV, vagal tone, or PNS function per se. The authors reported reduced heart rate to the stressor in children with ASD with co-occurring high anxiety compared to children with ASD and low anxiety, which they interpreted as "stress induced physiological withdrawal" (Mikita et al., 2015, p.1124). Finally, Hollocks and colleagues (2014) employed a variant of the TSST in a mixed sample of ASD children and adolescents with and without comorbid anxiety disorders. The authors examined the

ratio of low-frequency heart rate fluctuation to high-frequency heart rate fluctuation (LF/HF), a measure of the relative contribution of sympathetic arousal to HRV. The authors report group differences in HRV and LF/HF, with the ASD participants with co-occurring anxiety showing a blunted HRV response, but no difference between the ASD participants without anxiety and the TD control group. Hollocks and colleagues (2014) also report a group effect on heart rate, such that the ASD group with co-occurring anxiety disorders showed heightened heart rate at baseline and throughout the task compared to the ASD group without anxiety and to the TD group. Importantly, these studies employed adapted versions of the TSST that ostensibly tax visual attention and memory and do not use a social peer. Despite these differences from the current protocol, the findings of blunted ANS measures in ASD, outlined above, are in agreement with the findings in the present study (Levine et al., 2012; Hollocks et al., 2014; Mikita et al., 2015).

We report significant correlations between internalizing symptoms and anxiety problems and RSA. Previous investigations of children with ASD have also reported significant relationships between cardiac-neural measures of stress responsivity and parental reports of anxiety symptoms (Guy et al., 2014; Hollocks et al., 2014; Mikita et al., 2015). To date, the anxiety disorders literature suggests lower RSA at baseline in adults with post traumatic stress and panic disorders but not specific phobias compared to controls (for review see Friedman, 2007; Licht et al., 2009). There have not been reported differences between adults with anxiety disorders and those without in response to the TSST (Klumbies et al., 2014). Studies employing emotional regulation or trauma recall tasks in adolescents with anxiety disorders have shown no differences in RSA responsivity (Fisher et al., 2013; Shenk et al., 2014; Kirsch et al., 2015) or have found differences in female, but not male adolescents (Hastings et al., 2014). To date, there have been no studies employing the TSST in adolescents with anxiety disorders using RSA as an outcome measure. When compared to the findings of a correlation between RSA and anxiety symptoms in ASD, this suggests that anxiety may manifest differently physiologically in ASD than in individuals with anxiety but without co-occuring developmental disabilities. There is a need for comparison studies of individuals with ASD and co-occuring anxiety disorders and those without, as well as greater study of the way that anxiety manifests specifically in ASD versus typical development.

Pubertal development plays an important role in the physiological response to stress, both in terms of the development and stabilization of biological systems underlying the stress response, but also regarding the increased salience and complexity of social stimuli during adolescence, which is defined by the onset of puberty (Sisk and Foster, 2004; Gunnar et al., 2009). Studies of RSA in the general population confirm the importance of adolescent development physiologically and psychosocially (Woodall and Matthews, 1993). There is also evidence implicating sex differences in RSA, including greater variability in adult females than adult males (Snieder et al., 2007), which is consistent with the findings of the present study of adolescents. Future studies should clarify the contributions of sex steroid hormones during puberty to ANS function, reactivity, and stabilization by carefully accounting for sex steroid levels and, in females, the role of the menstrual cycle in physiological regulation and reactivity. Studies in the general adult population have indicated that there are both sex differences and menstrual cycle effects on the physiological response to social evaluative threat. Specifically, studies have shown greater heart rate response and subjective feelings of anxiety to the TSST during the luteal phase, suggesting that some aspects of the stress response may be mediated by progesterone levels (Childs et al., 2010; Gordon and Girdler, 2014). Furthermore, for adolescent girls with ASD, social deficits may become more pertinent in adolescence than they are for male adolescents, because social relationships may be more challenging, demanding, and complex for adolescent girls than they are for males (McLennan, et al., 1993). In one study of age- and IQ-matched adolescent boys and girls with ASD, there were not significant differences in core symptoms of autism, but the parents of girls with ASD were more likely to report significant social deficits via the CBCL than the parents of boys. Parents of the girls in this study were also less likely to report peer friendships than the parents of boys. The authors interpret these findings by suggesting that the parents of girls may have higher expectations for social competence than the parents of boys, and that these heightened expectations are mirrored by peers in the school environment, as well (Holtmann et al., 2007). Particularly in ASD, where females are more likely to be misdiagnosed or diagnosed later in life (Russell et al., 2011), there is a clear need for research that focuses on the experiences and needs of adolescent girls with ASD, especially as they are related to understanding and improving social abilities.

Despite the careful characterization of the sample in the present study, there are some limitations. The findings in the present study may not extend to other subpopulations within ASD. For

example, we were limited in our ability to address RSA findings in adolescent females due to the greater variability within the ASD cohort. Future studies that include a large sample of adolescent females are warranted to elucidate the mechanisms contributing to physiological variability in adolescent females with ASD. Future studies should also assess RSA in a more naturalistic, non-laboratory setting, as TD and ASD adolescents may differ in the degree to which they find the laboratory environment stressful (Corbett et al., 2012). Baseline measures of RSA taken at home in a familiar environment could provide an interesting comparison to RSA data collected in the lab. This study was part of a larger project that also investigated salivary cortisol response to the TSST. As such, participants taking medications known to interfere with HPA axis function were excluded during recruitment. Exclusion of adolescents taking medications such as atypical antipsychotics could potentially have biased our sample. Furthermore, because of the cognitively demanding nature of the TSST paradigm, we were not able to include individuals with intellectual disabilities, which also limits the generalizability of our findings.

To our knowledge, this is the first study examining RSA in adolescent males with ASD. The current investigation contributes to the growing literature on the biopsychosocial correlates of ASD in adolescence. The majority of previous work focusing on children has found reduced RSA in response to social stressors in ASD (Benevides and Lane, 2015). Although we did not find the hypothesized difference in variability between groups during the stressor, there was an overall group effect of reduced RSA in the ASD group, suggesting that reduced PNS reactivity in children persists into adolescence. Longitudinal studies that investigate the developmental effects of puberty on RSA are critical for an improved understanding of biopsychosocial development in ASD. Future studies should also compare types of potential stressors to determine if one type of stressor (i.e., social vs. nonsocial stress) may induce greater physiological effects in ASD.

CHAPTER IV

CONCLUDING REMARKS

In this study of male adolescents with ASD, we used three measures of arousal to determine effects of social judgment on individuals with ASD and TD during the TSST; these methods included behavioral, neuroendocrine (salivary cortisol), and physiological (RSA) measures. We found no significant salivary cortisol stress response in the ASD group. There was also reduced PNS function, as indexed by RSA, in the ASD group compared to the TD group. However, this difference was present at all time points and there were no significant group-by-time-period effects on RSA, indicating that differences in PNS function in ASD were not related to challenge or stress associated with the TSST task. We also found less displacement behaviors in the ASD group compared to the TD group. Taken together, these findings suggest that for adolescent males with ASD, social judgment did not activate the HPA axis, but did result in a decrement in PNS activity similar to that of TD participants. This seeming contradiction in findings, that the PNS responded to the TSST similarly in both ASD and TD groups, but the HPA axis did not, is best explained by the fact that these two systems regulate arousal and stress in different manners.

The HPA axis functions on a much more delayed time scale than the ANS. The HPA axis functions on an approximate lag time on the scale of minutes, with an estimated one-to-three minute lag from cortisol release to its presence in the periphery, and an approximate 20- minute lag from release to presence in saliva (Kirschbaum and Hellhammer, 1999). Thus, measurements obtained from cortisol are of a diminished temporal resolution compared to those obtained from ANS measurements such as RSA, which can be measured on time scales as small as one second (Berntson et al., 1993). Furthermore, RSA is thought to be an indirect measure of the regulatory capacity of the PNS (Porges et al., 1994), while the HPA axis is the primary driver of the stress response (Herman and Cullinan, 1997). Because in the present study RSA distinguished the groups at baseline, and the decrement in RSA between groups was similar at all time points of the stressor, we can preliminarily conclude that regulatory capacity is more closely related to basal function than to stress reactivity in ASD.

In this study, HPA axis and PNS function were reciprocally correlated at baseline in both TD and ASD adolescents. Because HPA axis function is closely coupled with SNS activity (Smith and

Vale, 2006), our baseline correlation finding would seem to indicate a reciprocal co-activation of these two systems at rest. However, this relationship was not present during the TSST, which could indicate differential co-activation of the PNS and SNS during response to a stressor in ASD. Interpretation of SNS function is outside the scope of the present study, but future studies should use impedance cardiography measures, such as pre-ejection period (PeP), to determine the relationship between the PNS and SNS responses to social stress in ASD. PeP would provide a more direct measure of SNS activity than salivary cortisol measures and allow for analysis of the coactivity of the PNS and SNS. To date, there have been no published studies of pre-ejection period in ASD (Benevides and Lane, 2015). Study of pre-ejection period would help to better determine if the differences in system responsivity reported in the present studies are due to a blunted "fight or flight" response in ASD, decreased salience of social stimuli, or if they are more closely mediated by overall poor physiological regulation or vagal tone.

The RSA findings in the present study are more closely linked to basal function than the stress task per se; our data demonstrate a correlation between parental report of social functioning, via the SRS total score, and baseline RSA in the ASD group. Furthermore, the importance of baseline arousal is supported by our findings of greater self-report of trait, but not state, anxiety in the ASD group, coupled with our preliminary finding of a correlation between parent-reported internalizing symptoms and RSA following, but not during, the TSST. More research is needed to elucidate the biological processes that differentially mediate anticipatory anxiety, basal physiological regulation, and physiological recovery or return to baseline in ASD. Regardless, these correlations suggests that in ASD, PNS function may be associated with a basal level of readiness to engage with the environment, and that PNS function does not differentiate ASD and TD adolescents during conditions of stress. This is in contrast to the broader literature in pre-pubertal children with ASD, which does not show group differences in RSA, but does show reduced or non-significant RSA response to challenge in ASD (Benevides and Lane, 2015). It is unclear whether the difference in the findings of the present study are due to the developmental effects of puberty; longitudinal studies of pre-pubertal children are needed to best determine the contribution of pubertal maturation to PNS response to social judgment. Furthermore, most previous work investigating RSA in children with ASD has used computer-based tasks that involve cognitive stressors (Althaus et al., 1999; Daluwatte et al., 2012) or social tasks that involve passive viewing of social actors on a screen (Bal et al., 2010; Watson et al., 2012). It is

possible that because the TSST necessitates real-time engagement with social peers, it may require differential allocation of physiological regulatory systems than passive viewing or computer-based tasks (Schilbach et al., 2012). Future studies should compare RSA responsivity in pre-pubertal and pubertal individuals with ASD to social judgment tasks that require social exchange to those that do not (Rolison et al., 2015).

There were no baseline differences between groups in salivary cortisol in either the samples taken on arrival before the TSST began or in the three days of home sampling. This stands in contrast to the differences in basal PNS function. It could be that the ability to apply a "vagal brake" is more related to clinical symptoms in ASD, such as social engagement, which is supported by our preliminary correlation analyses of parent self-report of social behavior in ASD. Conversely, HPA axis differences in ASD appear to be more closely tied to the stress response than to basal functioning, at least in the present sample of male adolescents. This is distinct from previous work in pre-pubertal children, which has found more variability in basal HPA axis function in ASD (Taylor and Corbett, 2014). It could be that by puberty, ASD adolescents have "caught up" with their TD peers in terms of diurnal and baseline HPA axis stability. Again, large-scale longitudinal studies are needed to better understand how pubertal development contributes to HPA axis function in ASD, as a thorough investigation of diurnal cortisol rhythmicity in adolescents with ASD was outside the scope of the present study.

The current study was underpowered to detect subgroups within the ASD sample in social behavior or co-occurring internalizing disorders and the relationship between social approach and arousal. It is clear that there need to be larger studies of the contributions of social anxiety to biobehavioral phenotypes across development in ASD. The behavioral component of the current study, although preliminary, indicated group differences in self-soothing displacement behaviors, and a correlation between frequency of these behaviors and salivary cortisol levels in the TD but not the ASD groups. It could be that, for individuals with ASD, there was a reduced incidence of self-soothing behaviors because the TSST was not perceived as stressful, although both ASD and TD groups indicated significant anxiety via self-report measure following the task. Conversely, it is possible that the blunted cortisol response is due to hypocortisolemia in the ASD group (Martí and Armario, 1997). Previous studies in our lab have indicated a blunted cortisol response to the TSST in pre-pubertal children with ASD (Lanni et al., 2012) and a heightened response to open-

ended play with novel peers (Corbett et al., 2010; Corbett et al., 2012; Schupp et al., 2013; Corbett et al.; 2014). As in child studies, this would indicate that the TSST is not stressful or salient compared to less structured interactions with peers for pre-pubertal children. In order to determine if the same can be said for pubertal adolescents, more study of the stress response in developmentally appropriate, open-ended social situations with adolescent peers is needed; if such scenarios were able to elicit an increase in HPA response, this would suggest that the TSST is uniquely less stressful for adolescents with ASD.

Although the TSST is thought to be a classic laboratory paradigm of social judgment or social evaluative threat (Buske-Kirschbaum et al., 1997), it does not appear to be interpreted that way by individuals with ASD. Furthermore, if open-ended, less structured social situations elicit a more robust HPA axis response in children with ASD, i.e., free play on a playground with a peer (Corbett et al., 2010; Corbett et al., 2012; Schupp et al., 2013; Corbett et al.; 2014), it is unclear if this is because open-ended situations are themselves stressful or challenging for individuals with ASD, regardless of their social content (White et al., 2009), or if these social situations with peers elicit a stress response because they more closely mimic potential prior experiences with peer ostracism or bullying than the TSST (Schroeder et al., 2014; De la Iglesia and Olivar, 2015; Fisher and Taylor, 2015; Weiss et al., 2015). Future studies of ASD adolescents should assess for bullying or victimization experiences, as well as degree of insight into the social nature of the TSST, to better clarify potential underlying interpretations of PNS and HPA axis reactivity during the task. Additional studies that compared physiological responsiveness to constructed versus open-ended social interaction tasks, i.e. a comparison of HPA reactivity to an open-ended play paradigm versus the friendly version of the TSST, which employs warm and supportive raters instead of judgmental, unsupportive raters (Wiemers et al., 2013), could also help to better elucidate the lack of a stress response to the TSST in ASD.

We were unable to determine the effects of social judgment on female adolescents in the present study due to a high degree of variability within the physiological data for ASD females and the small size of our sample of female adolescents. Just as adolescents with ASD are understudied, females with ASD are not well-represented in the research literature, due in part to the increased prevalence of ASD in males (Williams et al., 2008). However, a better understanding of the experiences of adolescent girls with ASD is extremely important, particularly regarding social

judgment, as girls with ASD experience a great deal of relational aggression in adolescence (Cridland et al., 2014). We are unable to determine what may underlie the high variability in RSA and cortisol in our female ASD sample. It could be that phase of menstrual cycle contributed to the variability, but then we would expect to see similar levels of variability in the TD female group, as well. Previous reports have suggested differences in ASD symptom profiles in females with ASD compared to males, including more parental reports of social problems by adolescence for females (McLennan et al., 1993; Holtmann et al., 2007), although these differences may be driven by gender biases in parental report, as clinician ratings on ADOS do not appear to differ by sex (Holtmann et al., 2007). Review of ADOS, SCQ, and SRS total scores in the present study did not suggest differing variability or means than the ASD male participants (see Appendix). Ultimately, we were underpowered to detect diagnosis by sex interaction effects on RSA. With a sample of only eight female participants total, we were limited in our ability to draw conclusions about the nature of the high variability in the ASD sample and what might underlie it. Future studies that focus specifically on recruiting adolescent girls with ASD, and that control for menstrual cycle phase, will be important to improving our understanding of the stress response in this population.

These data suggest blunted overall PNS activity in ASD compared to TD adolescents, as well as a blunted HPA axis response to social judgment. Our RSA findings suggest reduced regulatory capacity in ASD as well as greater baseline variability in RSA, which could indicate resting or basal physiological variability as a biomarker in ASD (Haigh et al., 2015). Enhanced variability in ASD could explain the heterogeneity in symptom severity as well as clinical presentation in the disorder (APA, 2013). Increasingly, evidence points to widespread alterations in cortical responsivity in ASD; with theoretical models suggesting that ASD may be related to a fundamental difference in the ratio of cortical excitation and inhibition (Rubenstein and Merzenich, 2003) and/or reduced central coherence of brain functional networks (Belmonte et al., 2004). It is unclear how alterations in cortical activity in ASD may relate to the ANS and HPA axis findings in the present study, but it may be possible that reduced coherence of cortical activity disrupts regulatory projections from the cortex to the paraventricular nucleus of the hypothalamus, which is a major regulatory hub of both ANS projections to the sino-atrial node of the heart, as well as the HPA axis (Silverman et al., 1981). More research is needed to determine

the underlying cause of these differences, including the relationship between PNS and HPA axis activity in adolescents with typical development, as well as in individuals with ASD.

Better insight into physiological regulation in adolescents with ASD will help to inform intervention and accommodation strategies for clinicians, educators, and families who support ASD adolescents. Specifically, enhanced understanding of physiological arousal can help supportive adults advocate for individuals whose level of arousal may not be apparent from their behavior or who may not be able to readily communicate their stress level. A better grasp of the relationship between symptom profiles, physiological arousal, and behavior will also help clinicians determine the most appropriate forms of intervention for individual patients, i.e., combining behavioral and medication therapies. Because reduced regulatory capacity has been linked to disruptive behaviors and emotional regulation difficulties in the classroom setting, which are in turn associated with academic underachievement in ASD (Ashburner et al., 2010), our findings may help to support educators who work with individuals with ASD. Finally, enhanced understanding of physiologic arousal and social judgment may help promote self-awareness among adolescents with ASD so that they can better self-advocate as they engage with the environment and build skills for interacting with others in the dynamic social world.

APPENDIX

VARIABILITY OF RESPIRATORY SINUS ARRHYTHMIA DATA IN ADOLESCENT
FEMALE SAMPLE

Diagnosis		Min	Max	Mean	SD
TD N=4	Baseline RSA	5.66	6.54	6.17	0.369
	Prep RSA	5.66	6.17	5.91	0.207
	Speech RSA	4.71	6.64	5.67	0.819
	Math RSA	4.77	6.19	5.49	0.675
	Debrief RSA	5.52	6.05	5.73	0.227
ASD N=4	Baseline RSA	4.30	8.63	6.02	2.07
	Prep RSA	5.54	8.34	6.77	1.32
	Speech RSA	4.33	7.60	5.88	1.63
	Math RSA	4.11	7.57	5.79	1.80
	Debrief RSA	4.28	8.72	6.31	1.97

Appendix Table 1: Descriptive statistics of RSA values in female adolescent sample
TD=typically developing, ASD=autism spectrum disorder, SD=standard deviation.

Sex	ADOS Total (SD)	SCQ Total Score (SD)	SRS Total T Score (SD)
Male	12.77 (4.48)	19.83 (9.60)	74.50 (10.76)
Female	11.75 (3.30)	19.60 (11.17)	84.20 (8.32)

Appendix Table 2: Descriptive statistics of clinical variables in ASD sample by sex
ADOS=Autism Diagnostic Observation Schedule, SCQ= Social Communication Questionnaire, SRS=Social Responsiveness Scale, SD=standard deviation.
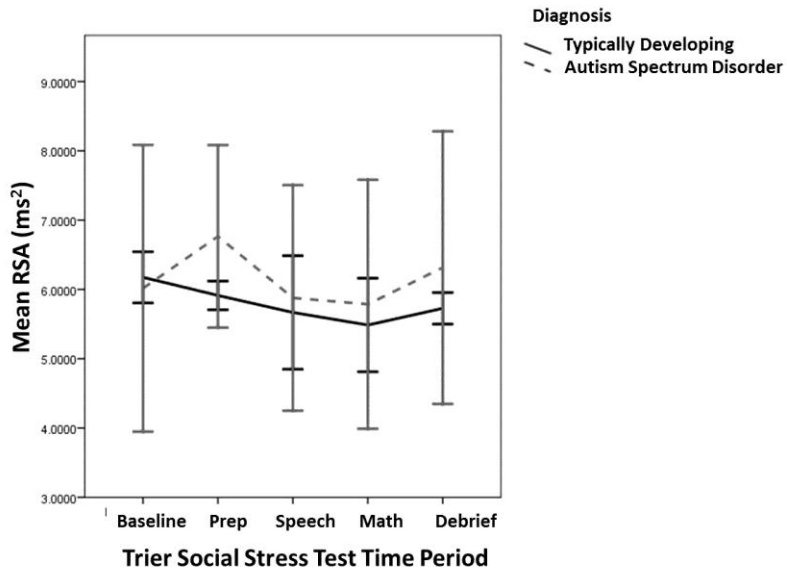
Appendix Figure 1: Increased variability in Respiratory Sinus Arrhythmia among females with ASD ASD=Autism Spectrum Disorder, TD=Typical Development, RSA=Respiratory Sinus Arrhythmia. Error bars represent SE.

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