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The Emergence and Predictive Utility of Early Markers of Social Anxiety in Young Children With Fragile X Syndrome

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THE EMERGENCE AND PREDICTIVE UTILITY OF EARLY MARKERS OF SOCIAL ANXIETY IN YOUNG CHILDREN WITH FRAGILE X SYNDROME

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DEDICATION

I would like to dedicate this dissertation document to my parents, who, from a young age, stressed the importance of being inquisitive, and provided the resources and experiences to thrive as a scientist, clinician, and person.

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ABSTRACT

Fragile X syndrome (FXS) is a monogenic neurodevelopmental disorder characterized by elevated rates of intellectual disability, autism, and anxiety. Social anxiety affects approximately 60 percent of individuals with FXS and is cited as one of the most impairing comorbid disorders in FXS; however, little is understood about the developmental patterns of social anxiety markers in young children with FXS. The current study investigates cardiac and behavioral markers of social anxiety across the first five years of life and tests the predictive utility of these variables on social anxiety symptomatology. Participants included 80 children with FXS and 50 neurotypical controls. Participants were tested multiple times between 3 and 60 months of age, resulting in 417 observations. Cardiac indices were measured at baseline and in response to a novel person. Behavioral measures were measured via parent report and direct observation. Our findings reflect complex relationships among and between physiological and behavioral indices of later-emerging social anxiety across early development in FXS. Results demonstrated that social behavioral inhibition was related to autonomic nervous system functioning in both groups. Additionally, baseline RSA was lower, and parent-reported social behavioral inhibition was higher by the third year of life in the FXS group compared to controls. The initial level and increasing rate of parent-reported social behavioral inhibition across age predicted social anxiety symptoms at preschool age. In contrast, RSA reactivity to a social challenge did not differ across groups and was not a predictor of social anxiety symptoms. These findings have

important clinical implications suggesting that early and repeated measurement of behavioral inhibition may aid in identifying social anxiety in young children with FXS as it has for neurotypical children. Given the elevated rate of social anxiety and its negative effect on the quality of life for children and adults with FXS, early identification and treatment of initial symptoms could result in the prevention of social anxiety later in life or reduced symptom severity.

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CHAPTER 1

INTRODUCTION

Social anxiety is a persistent, intense fear of negative evaluation, humiliation, or embarrassment in social situations that affects up to 20 percent of otherwise neurotypical children (Costello et al., 2005; Franz et al., 2013). In addition to high prevalence in neurotypical populations, social anxiety is common and impairing in specific neurodevelopmental disorders. Fragile X syndrome (FXS) is a monogenic neurodevelopmental disorder that results in intellectual disability and frequent co-occurrence with other psychiatric disorders, including anxiety (Cornish et al., 2008; Hagerman & Hagerman, 2002; Wheeler et al., 2014). Given the complex phenotype and overlapping symptom presentation of multiple disorders in individuals with FXS, the diagnosis of social anxiety is often nuanced, requiring multiple measures. Thus, the current study utilizes a multimethod approach that includes both cardiac indices and behavioral measures (biobehavioral) to examine the emergence and relations of early markers to social anxiety symptoms and diagnostic outcomes in FXS.

Social Anxiety in Neurotypical Children

Social anxiety is a prevalent impairing disorder in neurotypical individuals that can result in poorer relationships, poor academic performance, and an increase in suicidality (Bijl & Ravelli, 2000; Davidson et al., 2011). Notably, multiple early markers of social anxiety are present within the early developmental period. Despite the

identification of early markers, the average age of diagnosis is in early adolescence (Grant et al., 2005), suggesting that additional research is needed that can lead to earlier identification which in turn may help reduce the debilitating effects of social anxiety.

A normative developmental pattern of fear in response to a novel person exists, such that increased fear emerges in late infancy and diminishes by toddlerhood. Importantly, early behavioral and cardiac markers of social anxiety have been identified in infancy that often presents more severely than expected in normative development (Brooker et al., 2013). Specifically, high and stable behavioral inhibition, defined by excessive fear and shyness in response to novelty (Hirshfeld-Becker et al., 2008), is a robust predictor of concurrent and later social anxiety in preschool and elementary-aged children (Brooker et al., 2013). The relationship between high behavioral inhibition and later social anxiety symptoms suggests that behavioral inhibition may manifest at higher rates than expected in neurotypical development in infants at risk for social anxiety.

Another early behavioral marker of social anxiety is atypical gaze. The relations between social anxiety and gaze are more nuanced, demonstrating differing relations based on the severity and type of anxiety. The relations are such that increased gaze toward threatening social images is associated with severe generalized anxiety disorder. In contrast, increased gaze away from threatening social images is associated with social anxiety (Waters et al., 2008, 2010). This indicates that the pattern of gaze may differentially identify risk for social anxiety later in life. In sum, multiple early behavioral markers of social anxiety have been identified that persist into childhood.

In addition to behavioral markers, the autonomic nervous system (ANS) has been implicated as contributing to the behavioral manifestation of social anxiety. These findings are consistent with neurovisceral integration theory, which postulates that the central autonomic network, which includes specific brain regions and nerves responsible for ANS control, is related to numerous behavioral manifestations of psychopathology, such as executive functioning, regulation during social interaction and challenge, and temperament characteristics (Beauchaine & Thayer, 2015). One non-invasive method to measure ANS functioning is through the utilization of cardiac indices. The most widely used cardiac indices in social anxiety research are inter-beat interval (IBI) and respiratory sinus arrhythmia (RSA), or the beat-to-beat variation in heart rate that corresponds with breathing. IBI measures the mean time between heartbeats and is an index of overall ANS functioning, taking into account both the parasympathetic and sympathetic branches of the ANS (Prokhorov et al., 2021). RSA is a measure of heart rate variability and is considered an index of parasympathetic nervous system function. Given the measurement of both RSA and IBI, sympathetic nervous symptoms functioning can be indirectly examined when interpreting results from IBI and RSA together. Both IBI and RSA can be utilized to index an individual's ANS functioning during a baseline period and in response to a social stressor as an index of emotion regulation, which may exacerbate the risk for social anxiety.

Research examining relations between the ANS and social anxiety symptoms has demonstrated that atypical cardiac responses (i.e., no response or a large response) in reaction to a novel social situation (Alkozei et al., 2015; Beauchaine et al., 2019; Viana et al., 2017), as well as atypical ANS functioning during rest, is related to increases in

anxiety symptomatology (Hastings et al., 2008; Kemp et al.,2014). For example, children with higher rates of behaviorally measured anxiety during a social task showed a blunted cardiac response and took longer to recover from a social stressor than children with lower anxiety levels during the same task (Alkozei et al., 2015). RSA has also been utilized as a predictive marker of future social anxiety symptoms. Research has shown that RSA suppression in response to a stranger at six months of age was predictive of social anxiety symptoms in preschool (Brooker et al., 2013). Thus, ANS dysfunction is related to and potentially influences the behavioral manifestations of social anxiety.

The presence of early behavioral and cardiac markers of social anxiety in neurotypical children highlights the importance of taking a biobehavioral approach to identify potential prodromal symptoms of social anxiety. The early identification of social anxiety can result in the implementation of highly efficacious pharmaceutical and behavioral interventions to ameliorate the adverse effects of social anxiety in children (Edwards et al., 2010; Kennedy et al., 2009; Mayo-Wilson et al., 2014). For example, Kennedy and colleagues (2009) found that a brief, low-cost parent-mediated intervention in preschool had long-lasting implications for reducing symptoms associated with social anxiety.

Fragile X syndrome

FXS is a rare genetic disorder caused by a mutation of the CGG trinucleotide repeat on the X chromosome resulting in the methylation of the *Fragile X Messenger Ribonucleoprotein* (FMR1) gene (Garber et al., 2008). The methylation of the *FMR1* gene results in the altered production of *FMR1* protein, affecting brain structure and

function. Importantly, *FMR1* protein production varies across individuals creating a spectrum of symptoms and behaviors associated with FXS (Hagerman & Hagerman, 2002). Additionally, the X-linked nature of FXS results in sex-based differences, with males often displaying more significant impairment than females across the lifespan (Bartholomay et al., 2019).

FXS has multiple hallmark phenotypic features that are present throughout the lifespan. The most common impairing features include mild to moderate intellectual disability, autism spectrum disorder, and anxiety (Cordeiro et al., 2011; Cornish et al., 2008; J. E. Roberts et al., 2020). Intellectual disability affects almost all males and approximately 25 percent of females with FXS (Cornish et al., 2008). Autism spectrum disorder is diagnosed in approximately 50 to 60 percent of individuals with FXS (Kaufmann et al., 2017; Roberts et al., 2020). Additionally, 86 percent of children and adults with FXS meet diagnostic criteria for at least one anxiety disorder (Cordeiro et al., 2011; Ezell et al., 2019). Due to the overlapping symptom presentation of these three impairing conditions, the diagnosis of social anxiety is complex, often requiring multiple measures to identify specific behavioral features associated with social anxiety.

The manifestations of behaviors associated with social anxiety have been attributed to elevated physiological arousal suggesting that ANS dysfunction is an underlying mechanism associated with anxiety in FXS (Cohen, 1995; Hall et al., 2009; Klusek et al., 2015; Tonnsen, Shinkareva, et al., 2013). Importantly, differences in ANS functioning have been identified both at times of rest (Hogan et al., 2021) and in response to a social stressor (Black et al., 2021; Heilman et al., 2011; J. E. Roberts et al., 2001). Increased arousal due to ANS dysfunction has been implicated in contributing to and/or

maintaining the presence of social anxiety symptomatology associated with FXS (i.e., social avoidance, behavioral inhibition; Heilman et al., 2011; Roberts et al., 2001). For example, Heilman and colleagues (2011) found that the FXS group did not demonstrate the expected decrease in RSA in response to a social challenge demonstrating an atypical ANS response. More broadly, ANS dysfunction has been implicated as a transdiagnostic biomarker across multiple other clinical populations (i.e., social anxiety, autism, depression, ADHD; Beauchaine & Thayer, 2015; Brooker et al., 2013). Thus, the predisposition to ANS dysfunction may contribute to the comorbid conditions present in the FXS phenotype.

Social Anxiety in FXS

Social anxiety is a highly prevalent comorbid condition in FXS, affecting up to 75 percent of individuals. (Bartholomay et al., 2019; Cordeiro et al., 2011; Ezell et al., 2019; Groves et al., 2022). Notably, despite considerably less research examining social anxiety in females with FXS, results suggest that females may have slightly lower rates of social anxiety compared to males, ranging from 30-56 percent (Bartholomay et al., 2019). Importantly, this is still considerably higher than the NT population. In addition to being highly prevalent, social anxiety has been reported as one of the most impairing phenotypic features in FXS (Bailey et al., 2008; Wheeler et al., 2014). The presence of social anxiety in FXS has been associated with increased problem behavior, lower quality of life for the family, and less adaptability across environments (Bailey et al., 2008; Wheeler et al., 2014). The current body of research has identified early markers of social anxiety but has not explicitly examined how these early markers relate to later social anxiety.

Early Behavioral Markers of Social Anxiety in FXS

Behavioral inhibition is often utilized in studies examining early markers of social difficulty in infants and preschoolers with FXS. This body of research measures behavioral inhibition using Parent-reported and direct observation. Direct observation often measures behavioral inhibition by examining and coding specific behaviors associated with behavioral inhibition, such as facial fear, distress vocalization, escape behavior, or bodily fear.

Collectively, studies of behavioral inhibition in infants and preschoolers with FXS suggest that behavioral inhibition may be blunted in infants with FXS and transition to hyperresponsivity as children with FXS age into preschoolers. For example, in infants and preschoolers, two studies found similar or decreased behavioral inhibition in comparison to neurotypical peers (Black et al., 2021; Tonnsen et al., 2017), whereas, in preschoolers with FXS, a developmental effect emerged with increased behavioral inhibition associated with older age (Roberts, Crawford, Hogan, et al., 2019; Tonnsen, Shinkareva, et al., 2013) Specifically, increased facial fear was associated with older ages in the FXS group, but not the neurotypical controls (Tonnsen, Shinkareva, et al., 2013). Heightened facial fear at older ages was also found in a study examining behavioral inhibition within the first minute of interaction with a researcher (Roberts, Crawford, Hogan, et al., 2019). Moreover, longitudinal trajectories of distress vocalizations predicted later social withdrawal symptoms (Tonnsen et al., 2017). Taken together, these research studies highlight potential developmental changes in behavioral inhibition, highlighting the need to examine the longitudinal trajectories of behavioral inhibition.

In addition to more broad indicators of behavioral inhibition, developmental patterns in gaze have emerged with a change from increased gaze towards the novel person to away from the novel person. In infancy, the FXS group spent a significantly longer time looking toward the novel person compared to the neurotypical group (Black et al., 2021). Another paper examining gaze in 24-month-old participants found similar rates of socially avoidant eye gaze across toddlers with FXS, Down syndrome, and neurotypical groups. In preschoolers around 4.5 years of age, Scherr et al. (2017) found that the FXS group spent significantly less time looking toward the stranger than the neurotypical controls with similar rates to the idiopathic autism group. These studies highlight a potential developmental shift in gaze away from a novel person. To help elucidate this potential developmental shift, a set of longitudinal studies examined eye gaze within the first minute of interaction for infants and children with FXS compared to their neurotypical controls (Roberts, Crawford, Hogan, et al., 2019; Roberts, Crawford, Will, et al., 2019). Findings demonstrated that age was associated with decreased eye gaze in both groups. Additionally, the FXS group displayed less eye gaze than the neurotypical controls. However, eye gaze was not related to an overall anxiety symptom measure (Roberts, Crawford, Will, et al., 2019). Taken together, the pattern of eye gaze shows a developmental pattern from infancy through early childhood such that in infancy, increased or normative gaze towards the novel person is reported, whereas as children get older, increased gaze away from the novel person is reported.

Early Cardiac Markers of Social Anxiety in FXS

Baseline cardiac functioning has been implicated as an early marker and concurrent predictor of social difficulty in FXS. However, the body of research that

examines baseline cardiac functioning in relation to social anxiety is limited. In infancy, infants with FXS did not demonstrate differences in baseline RSA compared to neurotypical peers (Black et al., 2021). Notably, there were no significant relations between baseline RSA and parent-reported social behavioral inhibition, observed social behavioral inhibition, or gaze. In older children and adolescents, Hall and colleagues (2009) demonstrated that males and females with FXS had lower RSA at baseline, while only males with FXS exhibited shorter IBI than their neurotypical same-sexed siblings. To date, one longitudinal paper has examined baseline cardiac functioning that supported a developmental shift with differences in RSA and IBI by 24 and 29 months of age, respectively. Notably, shorter IBI at 24 months of age and a smaller rate of change over time were related to overall anxiety symptoms. In contrast, lower RSA at 24 months of age was not predictive of overall anxiety symptoms. In sum, cardiac indices of baseline ANS functioning may change across development and uniquely predict different aspects of social functioning.

Changes in cardiac activity between baseline and in response to a novel person, reactivity, have been measured across multiple studies examining overall anxiety in children with FXS. Specifically, in 12-month-old infants with FXS, a lack of RSA reactivity was observed in response to a novel person, which was unrelated to any concurrent behavioral measure (Black et al., 2021). These results contrasted with the neurotypical control sample, which showed significant reactivity of RSA from baseline to the novel person (Black et al., 2021). In early childhood, increased cardiac activity during a baseline period and in response to a social stressor was observed, suggesting an overall level of hyperarousal within FXS (Roberts et al., 2001; Tonnsen et al., 2013).

Importantly, cardiac levels were related to distress vocalization, a measurable construct component of behavioral inhibition, such that at younger ages, increased arousal was associated with increased distress vocalization. In comparison, increased arousal was associated with decreased distress vocalization at older ages. The authors posit that the changes in the relationship between arousal and distress vocalizations may be related to the known arousal shift during infancy for individuals with FXS (Tonnsen, Shinkareva, et al., 2013). Despite this, these studies do not examine how an atypical cardiac response is related to later social anxiety, just concurrent behavioral markers.

In older children and adolescents with FXS, a novel social task examined children's cardiac responses to a one-on-one conversation while the examiner was sitting close to the child. The child would be reminded to answer the questions and provide eye contact (Hall et al., 2009). The findings of this study demonstrate significantly lower vagal tone in individuals with FXS than their neurotypical siblings, but only the males demonstrated shorter IBI compared to their siblings. Interestingly, cardiac indices were not related to eye gaze in males or females (Hall et al., 2009). Overall, this study demonstrates significant differences in response to a social challenge task between FXS and neurotypical siblings.

Summary of Early Markers of Social Anxiety in FXS

The development of early markers of social anxiety in infants and young children with FXS may present with a lack of hyperresponsivity early in development, with hyperresponsivity emerging between 24 and 36 months. This is supported by behavioral and biological markers, which demonstrate a divergence from neurotypical controls

within the second year of life. However, in order to understand the specific developmental pattern, studies examining the longitudinal trajectories of early markers of social anxiety are needed to determine when markers diverge from neurotypical development.

Rationale for the Current Study

Social anxiety is a debilitating disorder that affects a high proportion of individuals with FXS. While a handful of studies have identified early markers of social anxiety in males and females with FXS, no work has examined the longitudinal development and predictive utility of early markers of social anxiety to outcomes across early childhood. The current study utilized a longitudinal, biobehavioral approach to identify behavioral markers and cardiac indices of social anxiety across early development in infants and preschoolers with FXS. A biobehavioral approach includes multiple measures that examine both behavioral (parent-reported behavioral inhibition, observed behavioral inhibition) and biological (baseline and reactivity of cardiac indices) constructs. The utilization of a biobehavioral approach is imperative for multiple reasons. First, recent research has determined a strong link between biological functioning and behavioral manifestations of social anxiety. Second, ANS dysfunction is well documented in FXS and suggests that behavioral manifestations in FXS may be directly related to, or caused by, underlying biological functioning. Thus, integrating biological and behavioral features in a single study allows a more nuanced understanding of the independence and association between these variables and how they signal risk for concurrent or future impairment.

Aims and Hypotheses for the Current Study

Specific Aim and Hypothesis 1. Determine if early markers of social anxiety are related across development within the FXS and NT groups.

1b. Examine the correlation between parent-reported and observed social behavioral inhibition. Given that these measure the same construct but through different perspectives and time courses, we expect that they would be moderately related.

1a. Examine the relationship between cardiac and behavioral markers of social anxiety during the presence of a novel person. As posited by neurovisceral integration theory, we expect that higher social behavioral inhibition would be related to blunted cardiac reactivity in the presence of a stranger.

Specific Aim and Hypothesis 2. Identify the longitudinal trajectories of behavioral and cardiac markers of social anxiety in infants and preschoolers with FXS compared to neurotypical matched controls. As suggested by our work and others, we hypothesize a developmental shift within the second year of life for the FXS group, reflecting elevated cardiac dysfunction and social behavioral inhibition. This developmental shift would result in a divergence from NT development.

Specific Aim and Hypothesis 3. Determine the predictive utility of behavioral and cardiac markers across infancy to social anxiety outcomes at preschool in FXS. We hypothesize that early markers of social anxiety will be positively related to later social anxiety symptoms.

CHAPTER 2

METHOD

Participants

The current study included 130 participants drawn from two longitudinal studies on the early development of autism and anxiety in children with FXS. The first study (1R01MH090194-01A1) focused on early signs of autism across infancy from 6 to 72 months, while the second study (R01MH107573) documented the emergence and trajectory of anxiety across preschool from 36 to 72 months. The current study leverages data from these studies and includes a longitudinal sample of infants and young children with FXS (n=80) and chronological-age-matched neurotypical controls (n=50) who participated in one or both of these studies. Across these two studies, assessments took place at 6, 9, 12, 18, 24, 36, 48, 60, and 72 months of age. The successive longitudinal studies of the same cohort allow for the examination of trajectories of social anxiety from infancy through preschool. A total of 417 assessments are included in this study, with variation in the number of assessments per individual across groups due to enrollment age, retention, and missing data. Analytic approaches were utilized that allow for varying numbers of assessments per participant and across groups. While ideal to have data from all participants starting at six months of age and ending at 72 months, large samples of infants with FXS are incredibly challenging to ascertain and nearly impossible to follow over this length of time. As such, we included all data available for each participant at each age, which aligns with an accelerated longitudinal design that maximizes power to

address the series of research questions. The average age for study entry was 21.74 (18.93), and the average age for the oldest timepoint was 65.82 (16.81). The oldest timepoint was utilized as the outcome timepoint to examine social anxiety symptom severity. Specific participants' characteristics can be found in Table 2.1.

The full mutation of fragile X was confirmed via genetic records documenting greater than 200 CGG repeats. Neurotypical controls were included based on the absence of a family history of ASD or related disorders (e.g., tuberous sclerosis, Down syndrome), no seizure activity, and confirmation of typical development through study assessment described in more detail below. In both groups, exclusion criteria included a gestational age of fewer than 37 weeks, the presence of vision or hearing impairment, and lack of English proficiency within the home. Participants with FXS were recruited through a national registry and social media, while the neurotypical participants were recruited through local pediatricians' offices and social media.

Procedures

The Institutional Review Board at the University of South Carolina approved all existing

studies. The assessments included a battery of behavioral and physiological measurements, including measures of cardiac function, behavioral inhibition, anxiety, broad development, and autism spectrum disorder. Parent-reported measures were mailed to the participant's parent before the assessment and collected at the in-person assessment. Participants were tested in their homes or within the research laboratory. A

developmental summary and monetary compensation were provided to families for each assessment. A team of trained research staff completed assessments.

Measures

Social Behavior Inhibition

Parent-Rated Social Behavioral Inhibition. This study utilized the Rothbart Temperament Scales that were appropriate to the given age range. The Infant Behavior Questionnaire-Revised (IBQ-R; Gartstein & Rothbart, 2003) was collected for infants between 3 and 12 months of age, the Early Childhood Behavior Questionnaire (ECBQ; (Putnam et al., 2006) was collected for children between 18 to 35 months-of-age, and the Children's Behavior Questionnaire (CBQ; Rothbart et al., 2001) was collected on children between 36 and 60 months-of-age. Of importance, previous studies have collapsed data across multiple age-appropriate questionnaires (Pijl et al., 2019, Wall et al., 2019). All three of these questionnaires ask parents/caregivers to rate how often their child responds to various situations on a Likert scale from 1 to 7, with higher scores indicating a higher frequency of behavior. They were normed on a diverse neurotypical sample of mother-child dyads and produced satisfactory internal consistency. Notably, the scales have been utilized on various samples with neurodevelopmental disabilities (Elliott, 2006; Pijl et al., 2019; Roberts, Crawford, Will, et al., 2019; Roberts et al., 2014; Wall et al., 2019). For this study, we computed a social behavioral inhibition score reflecting fear in response to unfamiliar adults for the IBQ-R consistent with existing studies (Black et al., 2021; Brooker et al., 2013). This score consisted of an average of eleven items in which the participants had greater than six responses. The eleven items

included all of the questions under the Fear subscale that included a social component. In the ECBQ and CBQ, the fear questions that include a social element create their subscale, the Shyness subscale (Putnam et al., 2006; Rothbart et al., 2001).

Direct Observation: Social Behavioral Inhibition Composite. To assess observed social behavioral inhibition, the Stranger Approach task from the Laboratory Temperament Assessment Battery (Lab-TAB; Goldsmith & Rothbart, 1996) was utilized to measure response to a novel person. The task consists of three parts: (1) the approach of the stranger the participant (10 seconds), (2) the stranger kneeling in front of the participant with a neutral facial expression (120 seconds), and (3) the stranger withdrawing from the participant (10 seconds). The stranger was a trained female research assistant dressed in standard attire. Before the administration of the Stranger, the mother was directed to remain neutral to allow the child to respond as "naturally" as possible. For this study, the approach, kneel, and withdraw periods were collapsed to measure the impact of the stranger for the entirety of their presence. After the assessment was complete, videotaped recordings of the Stranger Approach task were coded for escape behaviors, distress vocalizations, bodily fear, facial fear, and visual attention using *Noldus The Observer XT* (version 10.0 software, Noldus Information Technology, Leesburg, VA, USA). Escape behavior, distress vocalizations, bodily fear, and facial fear were coded for both intensity and duration (for more details and a specific coding scheme, see Scherr et al., 2017). An observed social behavioral inhibition composite score was derived by taking the z-score of facial fear, distress vocalizations, escape behavior, and bodily fear and averaging them together. A data-driven approach was taken to increase the availability of data, allowing for the calculation of a score if three out of

the four behaviors were coded. Reliability was checked on 20% of videos, with Cohen's Kappa ranging from 0.82 to 0.91.

Direct Observation: Averted Gaze. A measure of averted gaze was derived through direct observation of behavioral responses during the Stranger Approach task (described in detail above). Gaze directed at the stranger was coded independently of the other behaviors and not included in the composite, given evidence that eye contact and gaze uniquely predict social avoidance and anxiety (Roberts, Crawford, Hogan, et al., 2019; Waters et al., 2010). The proportion of time spent averting gaze from the stranger was computed to create an averted gaze variable.

Cardiac Indices

Baseline IBI and RSA. Baseline heart activity was collected before the LabTab and consisted of an engaging video that did not include language and was neutral. Electrocardiogram (ECG) data were recorded using a telemetry system that recorded via two electrodes placed on the participant's chest with a sampling rate of 300Hz (Alive Technologies; CamNTech Ltd., Cambridge, UK). Trained research assistants visually inspected the ECG signal to identify and correct arrhythmias, false heart periods, and artifacts using CardioEdit software (brain-Body Center, University of Illinois at Chicago). Data were excluded if greater than 10 percent of the file was edited. RSA was extracted via CardioBatch software. CardioBatch samples sequential heart periods in 250ms epochs and then utilizes a 21-point moving polynomial algorithm to de-trend the data. Data were bandpass filtered to remove variance associated with respiration (.3-1.3

Hz for participants between 3 & 30 months, .24-1.04 Hz for participants from 31+ months) with RSA estimated by transforming the variance to its natural logarithm.

Reactivity IBI and RSA. Heart activity was also collected during the entirety of the Stranger paradigm and underwent identical processing procedures to the baseline heart activity. Both RSA and IBI reactivity was calculated to account for individual variation in baseline activity by subtracting RSA or IBI during the Stranger paradigm from baseline RSA or IBI, respectively.

Anxiety Outcome Measure

The Preschool Anxiety Scale (PAS; Spence et al., 2001) was utilized to examine a continuous measure of social anxiety symptom count and severity. The PAS is a 28-item Parent-report questionnaire designed for young children to assess anxiety and worry symptoms. The Social Anxiety subscale is a sum of 6 items with a range from zero, not at all true, to four, very often true, resulting in a range of 0-24. The raw score was used from the oldest time point because anxiety symptoms are known to manifest later in life, with the peak of diagnoses occurring in the adolescent period.

Covariates

Developmental Level. Developmental level was measured by the Differential Abilities Scale – 2nd edition (DAS-II; Elliot, 2007) for those older than 68 months or the Mullen Scales of Early Learning (MSEL; Mullen, 1995) for those 68 months or younger. Developmental level was estimated via the General Conceptual Ability (GCA) score, a global measure of a child's functioning. The GCA includes three subdomains: Verbal Reasoning, Nonverbal Reasoning, and Spatial Abilities. The Early Learning Composite

(ELC) score from the MSEL was used as an overall level of developmental functioning. The ELC includes four subtests: Visual Reception, Fine Motor, Expressive Language, and Receptive Language. This was collected at each assessment time point.

Autism Symptom Severity. The calibrated severity score (CSS) from the Autism Diagnostic Observation Schedule – 2nd edition (ADOS-2; Lord et al., 2012) was utilized as an overall measure of autism symptoms and severity. The ADOS-2 is a semi-structured play-based activity that is designed to assess symptoms of autism in children greater than 18 months of age. The CSS score ranges from 1-10, with higher scores indicating greater symptom count and severity. The ADOS-2 was completed at all assessments from 24 months through the last assessment time point. Lab reliable research staff completed all ADOS-2 administrations.

Statistical Analysis Plan

Preliminary Analyses. Data were analyzed to ensure they met the statistical assumptions needed to complete the specific analysis. Repeated measures correlations were computed between all key variables and potentially related phenotypic features in FXS (e.g., autism symptom severity and developmental level). Additionally, to control for the effect of potential covariates in specific aim 3, spearman correlations were computed between the social anxiety outcome variable, autism symptom severity, and developmental level. An independent sample t-test was completed to examine if there were sex differences in social anxiety outcome.

Specific Aim 1a. To determine if parent-reported and observed social behavioral inhibition were related within groups, repeated measures correlations were utilized.

Repeated measures correlations examine within individual associations when two or more time points are included within the data (Bakdash & Marusich, 2017).

Specific Aim 1b. To examine if the cardiac and behavioral markers of social anxiety during the Stranger were related within the FXS and NT groups, within-group repeated measures correlations were utilized.

Specific Aim 2. Multilevel modeling was utilized to examine early markers of social anxiety trajectories between children with FXS and age-matched neurotypical controls. For each specific marker, the samples were age-matched to ensure the least possible data loss. Age was mean centered at six months as this was the first time point of the collected data. Separate models were completed for parent-reported social behavioral inhibition, observed social behavioral inhibition composite, gaze aversion, baseline RSA and IBI, and RSA and IBI reactivity in response to the Stranger. Random slopes and intercepts were utilized due to expected individual variation in the level and trend of the early markers across development. Significant interactions were probed by recentering age to determine the specific age at which children with FXS differed from neurotypical controls.

Specific Aim 3. To examine the predictive utility of the behavioral and cardiac markers of social anxiety on social anxiety outcome data in children with FXS, a regression model was utilized for each early marker for a total of 7 models. Each marker (e.g., observed social behavioral inhibition composite) was entered into a multiple linear regression to examine the predictive utility on a PAS social anxiety raw score. Child-specific slope and intercept were extracted from the growth models from Specific Aim 2

and entered as dependent variables. Additionally, covariates with a significant correlation to the early marker were entered into each model accordingly. Collinearity was examined in each model via a variance inflation test (VIF). The slope was removed from the model if a VIF value was greater than 10. To examine which marker accounted for the most variance, a comparison between R-squared values of the multiple linear regression was completed.

Table 2.1 Participant Characteristics

	FXS (n = 80; 20 Females)	NT (n =50: 10 Females)
Age, M(SD)	34.80 (18.90)	30.50 (18.60)
First Assessment Age, M(SD)	28.32 (18.99)	17.89 (13.28)
Outcome Assessment Age, M(SD)	65.82 (16.81)	67.59 (16.65)
Number of Observations, M (SD), Range	2.45 (1.68), 1-6	2.47 (1.36), 1-6
Outcome Cognitive Standard Score, M (SD)	76.55 (21.52)	98.44 (12.83)
Outcome Anxiety Symptom Raw Score, M (SD), Range	3.46 (4.20), 0-21	3.18 (2.83), 0-9
Outcome Autism Symptom CSS, M (SD), Range	5.09 (2.82), 1-10	2.73 (2.23) 1-7
Race, %		
American Indian/Alaska Native	3.33	0
Asian	0	0
Black or African American	5	7.27
White	71.66	80
More than one race	20	9.09
Not Reported	0	3.63
Ethnicity, %		
Hispanic or Latino	6.66	5.45
Not Hispanic or Latino	93.33	90.90
Not Reported	0	3.63
Household Income, M (SD)	47,230 (78,511)	75,354 (44,712)

Note. CSS = ADOS-2 Calibrated Severity Score

CHAPTER 3

RESULTS

Descriptive statistics of all key variables can be found in Table 3.1. Repeated measures correlations between key variables and potential covariates were investigated in the FXS group to control for potential confounding effects in specific aim 3 (See Table 3.2). *Specific Aim 1a: Examine the relationship between parent-reported and behavioral measures of social behavioral inhibition.* Correlations can be found in Table 3.3.

FXS. Parent-reported social behavioral inhibition was related to averted gaze, r = .60, p < .001, but not the observed social behavioral inhibition composite, r = -.09, p = .77.

Neurotypical. Parent-reported social behavioral inhibition was related to averted gaze, r = .85, p < .001, but not the observed social behavioral inhibition composite, r = .28, p = .254.

Specific Aim 1b: Examine the relationship between early cardiac indices and behavioral measures of social anxiety during the presence of a stranger in young children. Figure 1 displays the within-group repeated measures correlations.

FXS. Observed social behavioral inhibition was significantly related to averted gaze, r = .63, p = <.001, and IBI reactivity, r = -.57, p = .002 but was not related to RSA reactivity, r =.21, p = .247.

Neurotypical. Observed social behavioral inhibition was not significantly related to averted gaze, IBI reactivity, or RSA reactivity, rs < .21, *p*s > .528.

Specific Aim 2: Examine the longitudinal trajectories of cardiac and behavioral markers of social anxiety.

Table 3.4 displays the fixed effects for all multilevel models discussed below. Figure 2 includes the dependent variable plotted across the first five years of life to illustrate the trajectories visually.

Parent-Reported Social Behavioral Inhibition. The results indicated nonsignificant main effects of age, t = -.44, p =.662., and group, t = .81, p =.423. A marginally significant age-by-group interaction emerged, t = 1.89, p =.065. The interaction was probed, and a significant group difference was observed starting at 39 months of age. Thus, higher social behavioral inhibition was observed in the FXS group from 39-60 months.

Observed Social Behavioral Inhibition Composite. Results indicated a significant main effect of age t = -2.11, p =.039. The observed social behavioral inhibition composite z score decreases by .07 per month. Group, t = -1.64, p =.105, and the group by age interaction, t = 1.62, p =.111, were not significant**.**

Averted Gaze. Results indicated a significant main effect of age, t = 5.20, p < .001. Averted gaze increased by .01 each month. The main effect of group, t = -.01, p =.996, and the group by age interaction, t = 1.27, p =.207, were not significant

Baseline RSA. Results indicated a significant main effect of age, $t = 11.87$, $p < .001$. The main effect of group was not significant, $t = -.42$ $p = .675$; however, the group-by-age interaction was significant, $t = -2.10$, $p = .028$. Specifically, for each one-month increase in age, the FXS group increased .01 less than the neurotypical group. The interaction was probed, identifying that the FXS group had significantly lower baseline RSA starting at 27 months, $t = -1.99$, $p = .049$.

Baseline IBI. Results indicated a significant main effect of age, $t = 13.56$, $p < .001$. The model displayed that for a one-month increase in age, the IBI increased by 3.59 milliseconds. The main effect of group, $t = -.07$, $p = .947$, and the interaction of group by age, $t = -1.23$, $p = .223$, were not significant.

RSA Reactivity. The main effect of age, $t = -1.61$, $p = .112$, group, $t = -1.51$, $p = .139$, and the age by group interaction, $t = .96$, $p = .341$, were not significant.

IBI Reactivity. A significant main effect of age, $t = 2.32$, $p = .022$, was observed. Specifically, for every one-month increase in age, IBI reactivity increased by .75 milliseconds. Group, $t = -.81$, $p = .419$, and the age by-group interaction, $t = 1.12$, $p = .264$, were not significant.

Specific Aim 3. *In the FXS group, examine the predictive utility of the early biobehavioral markers and the developmental change of these markers on later social anxiety.*

The social anxiety outcome measure was not related to autism symptom severity or developmental level, $rs < .12$, $p > .367$. Additionally, social anxiety symptomatology did not differ between males and females, $t = .57$, $p = .572$. To assess the predictive

utility of each early marker of social anxiety, a linear regression model was utilized. Table 6 displays the model results. The only early marker that was significantly predictive of social anxiety symptoms was parent-reported social behavioral inhibition, $F(2,48) = .48$, $p < 001$, $R^2 = .48$, slope, $t = 5.76$, $p < .001$, and intercept, $t = 3.04$, $p = .004$.

Table 3.1 Summary of Key Variables

	Fragile X Syndrome	Neurotypical
Parent-Reported Social Behavioral Inhibition		
M (SD)	3.77 (1.19)	3.31 (1.34)
Number of Participants, N	75	48
Total Number of Observations	145	112
Observation per Participant, M (SD)	2.75 (1.36)	2.96 (1.15)
Chronological Age (months), M (SD)	38.47 (17.18)	37.79 (17.51)
Observed Behavioral Social Inhibition Composite		
M (SD)	.11 (2.12)	.51 (2.02)
Number of Participants, N	39	39
Total Number of Observations	108	100
Observation per Participant, M (SD)	1.88 (.90)	1.91 (.80)
Chronological Age (months), M (SD)	16.80 (7.34)	15.07 (6.50)
Proportion of Averted Gaze		
M (SD)	.63 (.17)	.64 (.18)
Number of Participants, N	41	44
Total Number of Observations	119	119
Observation per Participant, M (SD)	1.88 (.99)	1.94 (.89)
Chronological Age (months), M (SD)	18.4 (9.07)	15.60 (6.77)
Baseline RSA		
M (SD)	4.98 (1.66)	5.32 (1.52)
Number of Participants, N	53	48
Total Number of Observations	157	154
Observation per Participant, M (SD)	2.96 (1.65)	3.21 (1.03)
Chronological Age (months), M (SD)	32.33 (19.63)	31.07 (19.02)
Baseline IBI		
M (SD)	534.20 (89.67)	542.51 (82.61)
Number of Participants, N	53	48
Total Number of Observations	157	154
Observation per Participant, M (SD)	2.96 (1.65)	3.21 (1.03)
Chronological Age (months), M (SD)	32.33 (19.63)	31.07 (19.02)
RSA Reactivity		
M (SD)	4.55 (1.37)	4.78 (1.40)
Number of Participants, N	49	48
Total Number of Observations	128	128
Observation per Participant, M (SD)	3.48 (1.46)	3.82 (1.52)
Chronological Age (months), M (SD)	33.65 (19.17)	33.28 (18.43)
IBI Reactivity		
M (SD)	495.37 (74.71)	507.22 (77.04)
Number of Participants, N	49	48
Total Number of Observations	128	128
Observation per Participant, M (SD)	3.48 (1.46)	3.82 (1.52)
Chronological Age (months), M (SD)	33.65 (19.17)	33.28 (18.43)

Table 3.2 Correlations between key variables and potential covariates in the FXS group

	Developmental level	Autism symptom severity
Parent Reported Social Behavioral Inhibition	-.17	.06
Observed Social Behavioral Inhibition Composite	-.27	-.06
Proportion of Averted Gaze	-46**	.07
Baseline RSA	-.33***	-.10
Baseline IBI	-.41***	-.16
RSA Reactivity	-.21	-.18
IBI Reactivity	-.22*	-.04
Social Anxiety	.06	-.12

Note. $p < .05$ = *, $p < .01$ = **, $p < .001$ = ***

Table 3.3. Biobehavioral correlations during the Stranger Paradigm

	1	2	3	4
Fragile X Syndrome				
1. Observed Social Behavioral Inhibition Composite	-			
2. Proportion of Averted Gaze	.63***	-		
3 RSA Reactivity	.28	.21	-	
4. IBI Reactivity	.60**	.40**	.83***	-
Neurotypical				
1. Observed Social Behavioral Inhibition Composite	-			
2. Proportion of Averted Gaze	.01	-		
3 RSA Reactivity	-.21	.62**	-	
4. IBI Reactivity	-.08	.44*	.74***	-

Note. $p < .05$ = *, $p < .01$ = **, $p < .001$ = ***

Table 3.4 Fixed effects of multilevel models

Predictor	β	*t*	*p*
Parent-Reported Social Behavioral Inhibition			
Centered Age (6 months)	-.00	-.44	.662
Group (Reference = TD)	.18	.81	.423
Centered Age:Group	.02	1.89	.065
Observed Social Behavioral Inhibition Composite			
Centered Age (6 months)	-.06	-2.11	.039
Group (Reference = TD)	-1.05	-1.64	.105
Centered Age:Group	.07	1.62	.111
Proportion of Averted Gaze			
Centered Age (6 months)	.01	5.20	<.001
Group (Reference = TD)	.00	-.01	.996
Centered Age:Group	-.00	-1.27	.207
Baseline RSA			
Centered Age (6 months)	-.06	11.87	<.001
Group (Reference = TD)	-.09	-.42	.675
Centered Age:Group	-.01	-2.21	.028
Baseline IBI			
Centered Age (6 months)	3.59	13.56	<.001
Group (Reference = TD)	-.64	-.07	.947
Centered Age:Group	-.45	-1.23	.223
RSA Reactivity			
Centered Age (6 months)	.01	1.61	.112
Group (Reference = TD)	-.42	-1.51	.139
Centered Age:Group	.009	.960	.341
IBI Reactivity			
Centered Age (6 months)	.75	2.32	.022
Group (Reference = TD)	-8.63	-.81	.419
Centered Age:Group	.50	1.12	.264

Table 3.5 Linear Regression Models

Predictor	*Estimate*	*SE*	*t*	*p*	R^2	F
Parent Behavioral Inhibition					.48	21.77
Model intercept	2.70	.47	5.74	<.001		
Intercept	5.90	1.94	3.03	.003		
Slope	264.76	45.90	5.77	<.001		
Observed Behavioral Inhibition**					.01	.135
Model Intercept	3.89	.74	5.23	<.001		
Intercept	-1.81	.45	-.72	.479		
Averted Gaze					.05	1.05
Model Intercept	3.52	.56	6.25	<.001		
Intercept	62.41	188.17	.332	.741		
Slope	-18.52	16.02	-1.15	.253		
Baseline RSA**					.03	1.07
Model Intercept	3.64	.67	5.39	<.001		
Intercept	1.07	1.03	1.03	.307		
Baseline IBI					.03	.62
Model Intercept	3.69	.68	5.41	<.001		
Intercept	.14	.13	1.10	.276		
Slope	-.39	.92	-.43	.670		
RSA Reactivity					.01	.26
Model Intercept	3.76	.72	5.26	<.001		
Intercept	-2.83	7.80	-.36	.720		
Slope	17.86	129.49	.14	.891		
IBI Reactivity*					.03	1.05
Model Intercept	3.76	.70	5.36	<.001		
Intercept	.25	.24	1.02	.312		

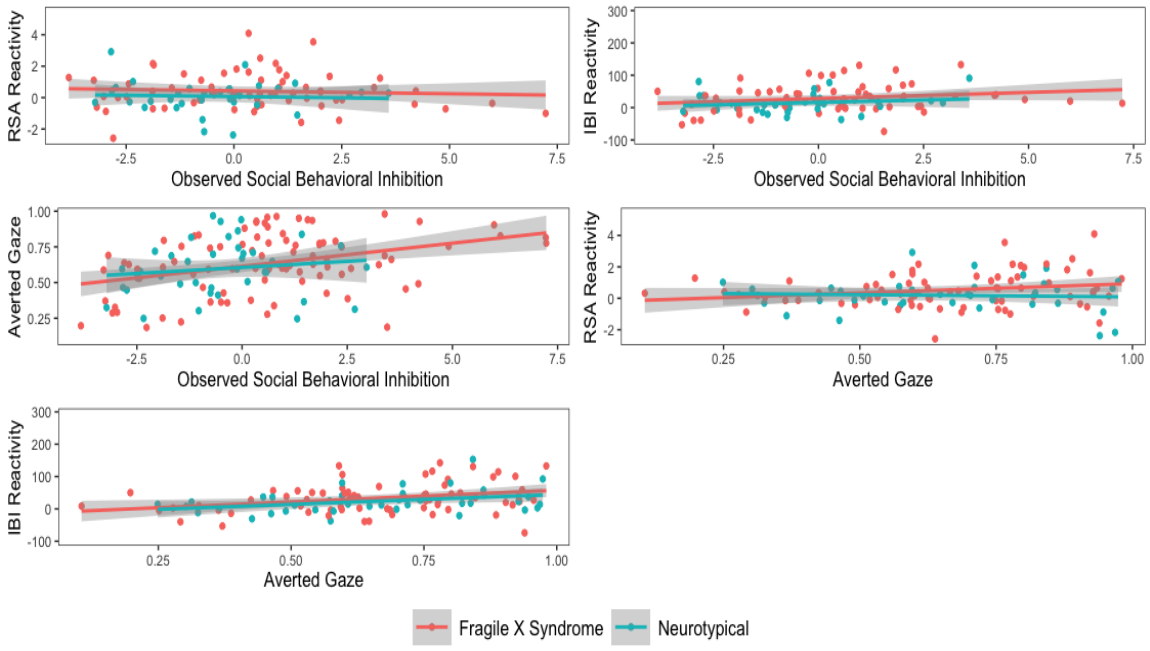

Figure 3.1 Correlations between cardiac and behavioral measures during the Stranger *Paradigm*

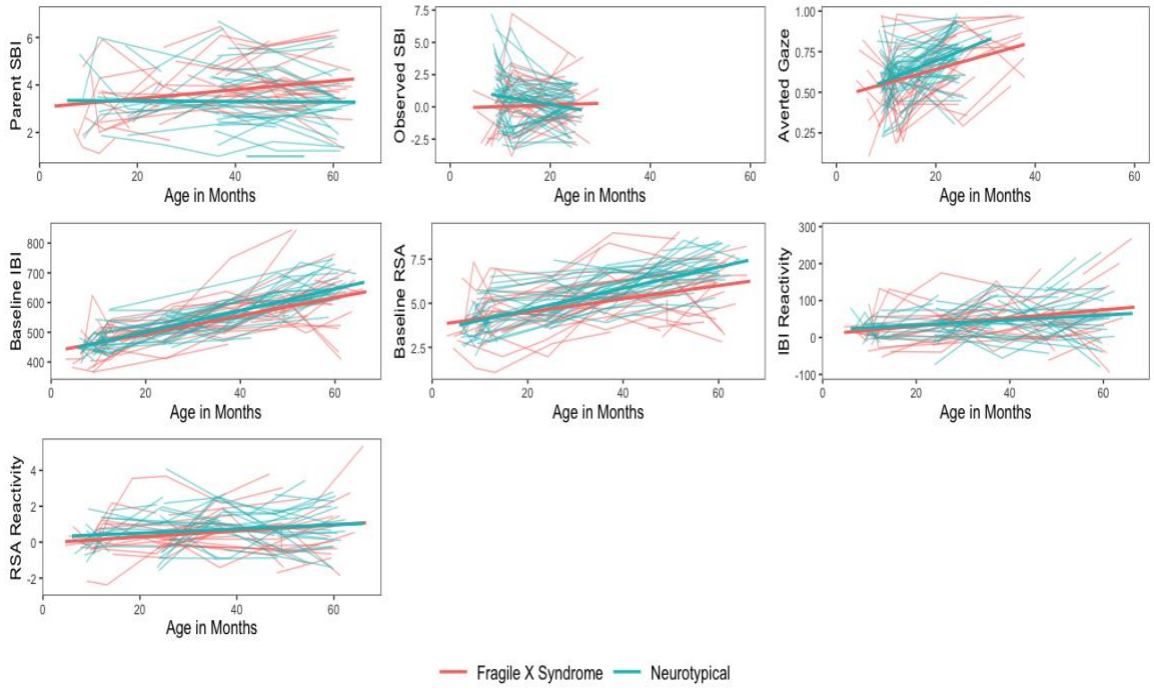

Figure 3.2 Early biobehavioral markers across time

CHAPTER 4

DISCUSSION

Social anxiety is a highly prevalent, impairing condition in individuals with FXS. The present study characterized the emergence and predictive utility of early markers of social anxiety in infants and young children with FXS. This work is critical to inform the timing and targets of intervention and as an aid in differential diagnosis. Findings suggest that early markers of social anxiety are measurable as young as six months of age and are predictive of later social anxiety symptoms at five years of age. This is the first study to take a biobehavioral longitudinal approach to examine early markers of social anxiety and determine their relationship to social anxiety symptoms later in life across early development in FXS. Evidence suggests meaningful relationships between behavioral and biological markers of social anxiety that are developmentally dynamic.

The measurement of social behavioral inhibition in young children with intellectual disabilities is complex, so the inclusion of multiple complementary measures is critical. The current study utilized both parent report and direct observation measures that examined social behavioral inhibition. These two discrete measures examined excessive shyness or fear in response to a novel person via gaze, body movement, vocalization, and escape behaviors; however, the context and time course were different. The parent-report measure asked parents to rate their child's social behavioral inhibition over the last couple of weeks in their typical environment. In contrast, the observed social

behavioral inhibition measure utilized a standardized, laboratory-based, intense social situation in the presence of a stranger, which was coded by a reliable research assistant across multiple different behaviors. Determining the relationship across these discrete measurements of social behavioral inhibition extends our understanding of how different facets of social behavioral inhibition develop over time and how they predict social anxiety. Results indicated that parent-reported social behavioral inhibition was discretely related to averted gaze but not the overall composite measure. One potential explanation of the observed difference may be due to the overt nature of averted gaze that is more readily observed by others, including parents, so a parent may naturally attribute averted gaze to social behavioral inhibition, whereas the observed social behavioral inhibition composite includes behaviors that may be less noticeable. For example, a child with FXS may have more subtle changes in facial expression to indicate fear and, thus, potentially not observed by the parent. Moreover, given that gaze avoidance is a hallmark feature of FXS, parents may be primed to look for this in their child.

Lower developmental level was associated with increased averted gaze but not with parent-reported or observed social behavioral inhibition in the FXS group. This suggests that the more severe cognitive impairment, the greater the proportion of time looking away from the novel person. Given the sample's age for averted gaze (Mage = 18.4 months), a lower cognitive level could reflect a young child being at the height of normative stranger fear, resulting in an increased gaze avoidance. Moreover, no relations between parent-reported and observed social behavioral inhibition emerged, contradicting previous research. Specifically, Tonnsen and colleagues (2013) found relations between developmental level and specific aspects of social behavioral inhibition (distress

vocalizations and facial fear). However, the current study did not find a relationship between social behavioral inhibition and developmental level which may be because we measured social behavioral inhibition more globally rather than examining each behavior individually.

The relations between cardiac and behavioral markers in response to a novel person have important differences between the NT and FXS groups. In the NT group, averted gaze was related to both IBI and RSA reactivity, which suggests that gaze was associated with both an overall measure of autonomic functioning and a measure of just the parasympathetic branch of the ANS. Interestingly, RSA and IBI reactivity was not related to the observed social behavioral inhibition composite, suggesting that increased autonomic arousal was not related to measurements of bodily fear or distress vocalizations. Potentially, this could highlight that eye gaze is more directly related to the autonomic nervous system than other measures of the social behavioral inhibition construct. The relationship between averted eye gaze and cardiac markers is consistent with previous literature that found relations between autonomic nervous system functioning and markers of social anxiety (Beauchaine & Thayer, 2015; Brooker et al., 2013). This may indicate that the behavioral paradigm accurately modeled a novel social situation in which NT infants and young children with higher levels of averted gaze demonstrated increased autonomic arousal, further supporting the autonomic nervous systems' implication in the development and maintenance as a transdiagnostic biomarker for psychopathology.

In the FXS group, this is the first study to examine the relationship between directly observed behavioral and cardiac markers in the presence of a novel person across

the first five years of life. Findings from existing cross-sectional research are mixed, with some studies reporting a relationship between behavioral and cardiac markers, whereas others have found no significant relationships. Hall and colleagues (2009) found no relationship between IBI or RSA and avoidant eye gaze during a social challenge task in adolescents with FXS. In contrast, higher distress vocalizations, a component of social behavioral inhibition, were related to higher RSA in the presence of a novel person in children around four years of age in other work (Tonnsen, Shinkareva, et al., 2013). In the current study, we found a strong relationship between avoidant eye gaze, observed social behavioral inhibition, and IBI reactivity, but not RSA reactivity. This may suggest that there is a stronger coupling of behavioral and cardiac indices at younger in life.

In addition, relations between cardiac and behavior differ in the FXS and NT groups. First, no relationship between RSA reactivity and social behavioral inhibition was observed in the FXS group, suggesting a lack of relationship between the parasympathetic nervous system and social behavioral inhibition. Second, IBI reactivity was strongly related to both the observed social behavioral inhibition composite and averted gaze. Given the lack of a relationship between the parasympathetic system and social behavioral inhibition in FXS, the strong relationship between IBI reactivity and social behavioral inhibition may be related to the sympathetic branch of the ANS. This aligns with the idea that the presence of a novel person is stressful, resulting in increased activation of the sympathetic nervous system. Taken together, these suggest that the reactivity of the ANS in response to a novel person and its relationship to social behavioral inhibition differ in FXS compared to NT controls, and that difference may be related to sympathetic activation.

Parent-reported social behavioral inhibition developmental trajectory was significantly steeper in the FXS group than in the NT controls indicating that the FXS group demonstrated significantly higher parent-reported social behavioral inhibition that emerged between 39 and 60 months of age. Thus, parents and caregivers rated similar rates of social behavioral inhibition during the infant and toddler ages. However, the steady increase across age in the FXS group resulted in a clear distinction by preschool. The developmental shift in social behavioral inhibition from mild to significant elevations in the FXS group has been reported in multiple cross-sectional studies in FXS. For example, Black and colleagues (2021) found parent-reported social behavioral inhibition that was similar between groups, while observed social behavioral inhibition was less in the FXS group compared to NT controls. In preschoolers, elevated social behavioral inhibition was observed in response to a novel person, such that higher levels of facial fear were present in the FXS group compared to the NT controls. These findings highlight a development shift in social behavioral inhibition between the 2 and 3rd years of life, which is consistent with our hypothesis and findings for parent-reported social behavioral inhibition. Notably, the lack of difference in observed behavioral inhibition may be related to the significantly younger mean age of the sample (FXS Mean Age = 16.80), in which, based on previous literature, we would not expect to see differences emerge in social behavioral inhibition until the latter ages in the sample. In sum, the current study highlights a developmental shift in social behavioral inhibition at approximately 36 months of age for individuals with FXS.

The divergence in parent-reported social behavioral inhibition between the FXS and NT groups occurring around three years of age coincides with the mean age of

autism spectrum disorder. Notably, parent-reported social behavioral inhibition was not related to autism symptom severity in FXS, suggesting that these profiles develop independently. The relations between behavioral inhibition, anxiety, and autism have been examined in previous studies examining a higher-level temperament characteristic, negative affect, which is related to behavioral inhibition (Tonnsen, Malone, et al., 2013; Wall et al., 2019). In these two other studies, negative affect was related to anxiety, not autism. Taken together, this suggests that the development of autism spectrum disorder and anxiety in FXS is independent and may represent differing risk factors specific to the disorder.

The current study suggests that baseline RSA is significantly lower in FXS contrasted to NT controls starting at 27-months-of-age whereas our previous work suggested that divergence began younger at 24-month-of-age (Hogan et al., 2021). For IBI, the current study suggests no significant difference at any age, whereas existing work reported shorter IBI in FXS that was evident starting at 29 months of age. Notably, the Hogan and colleagues (2021) sample overlaps with the current study sample (71 out of 101 participants). Key differences include a shorter age span in the current study (e.g., 6 to 60 months vs. 3 to 83 months) and the inclusion of females in this study, whereas Hogan and colleagues excluded female participants. To explore sex effects in our sample, we examined the effect of sex in multiple ways. First, an independent sample t-test did not show significant differences in IBI between males and females. Second, we ran analyses that included sex as a fixed effect which did not alter the primary findings. Third, we ran a multilevel that included FXS and NT males only, that entered age, group, and a group-by-age interaction predicting baseline IBI (same structure as the initial

model, just excluding females). A significant interaction emerged with the males with FXS having significantly lower IBI by 48 months of age, $t = 1.91$, $p = .049$. These findings suggest that females with FXS likely differ from males with FXS in the developmental trajectory of IBI. To date, one other study has identified differences in baseline cardiac functioning between males and females with FXS. Specifically, in adolescents, Hall and colleagues (2009) found no differences between FXS females and their sisters in baseline IBI. Taken together, this suggests that differences may be milder in females, which is consistent with the phenotype related to random X-inactivation (Bartholomay et al., 2019). The difference in findings between baseline RSA and IBI highlight the unique contribution of the parasympathetic and sympathetic branch of the ANS. Future studies should investigate parasympathetic, sympathetic, and an overall measure of autonomic nervous system functioning to better understand the potentially unique variation within each measure. Further, given the current findings, these research studies should be designed with adequate power to detect both group and sex differences.

While baseline RSA was lower in the group with FXS and baseline IBI was shorter in the males with FXS, neither IBI nor RSA reactivity (difference between baseline and reactivity to a novel person) trajectories differed between groups. This suggests that the ANS response of young children with FXS and NT controls to the presence of a novel person is similar. Previous cross-sectional studies in young children have found an overall level of hyperarousal with reactivity being significantly blunted in the FXS group compared to the NT controls (Hall et al., 2009; Hogan et al., 2021; Roberts et al., 2001; Tonnsen, Shinkareva, et al., 2013). In younger children, Tonnsen and colleagues (2013) examined RSA and IBI at baseline and in response to a novel

person in young FXS and NT males using an analytic approach that did not account for individual variation. The results found significantly lower RSA and shorter IBI at both baseline and in response to a novel person, but no group differences in RSA or IBI reactivity.). In older males and females with FXS, vagal tone was significantly lower in the FXS group compared to the neurotypical controls in a social challenge task where the examiner reminded the participant to make eye contact every 30 seconds. Together, these findings highlight that while there is a clear difference in ANS functioning between individuals with FXS and their NT controls, reactivity to a social situation may be similar in FXS and NT development in certain contexts or at specific ages. Alternatively, the large variation in responses across both groups could mask important potential subgroups at heightened risk for social anxiety or other psychiatric conditions.

Parent-reported social anxiety outcomes demonstrated a wide range of scores, with the overall mean relatively low and not significantly different across groups. Likewise, the rate of elevated social anxiety symptoms in the FXS group (T Score >60) was low with only 3 participants above this threshold (5.36 %). This suggests that the majority of individuals with FXS and the NT controls did not exhibit significantly impairing levels of social anxiety, which is consistent with developmental expectations. Of importance, social anxiety scores were not related to autism symptom severity or developmental level, suggesting that social behavioral inhibition is independent of ASD and ID within FXS. Given the overlapping symptom presentation between social anxiety and ASD, we expected a relationship between the two. The lack of relationship is supported by previous literature suggesting that social anxiety and ASD develop independently (Roberts et al., 2018; Tonnsen, Malone, et al., 2013; Wall et al., 2019).

The difference in development may be related to certain phenotypic differences between the two disorders. One key difference between the two phenotypes is social motivation. In social anxiety, children may actively avoid looking and interacting with a novel person initially, but this typically abates over time with increased familiarity. In contrast, children with ASD are typically more aloof and unmotivated to actively engage with social partners regardless of novelty or time spent with an individual. The difference in presentation is also supported by previous work that suggested prolonged social avoidance is related to ASD, whereas social avoidance within the first minute of the interaction was related to anxiety (Roberts, Crawford, Will, et al., 2019). Thus, individuals with FXS and social anxiety may be nervous at first but "warm up" after the initial interaction, which differs from individuals who have FXS and ASD, may never "warm up." This difference suggests that while both ASD and social anxiety are related to social deficits, the underlying mechanisms and associated behaviors are different. This lack of relationship is important because it highlights that these early markers are uniquely predictive of social anxiety and not influenced by ASD symptom severity.

Parent-reported behavioral inhibition during infancy was the only early marker that significantly predicted parent-reported social anxiety symptoms at preschool. This suggests that excessive shyness measured in six-month-old infants was measurable and predictive of later social anxiety symptoms. This finding highlights that parents are seeing observable behaviors that are indicative of social anxiety risk. Because the level of excessive shyness increased across the first five years of life, and that increase was predictive of social anxiety symptoms, this has important implications for understanding and tracking these early symptoms and outcomes. Taken together, this highlights the

value of a parent-report measure to both detect and monitor behavioral inhibition in FXS across the first 5 years of life. This has multiple critical clinical implications. First, given that it is a brief parent-report measure, it can be collected in a short window via an online rating form, in a telehealth visit, or even in the waiting room before the family sees the medical provider. The ease of collection and predictive utility of the measure could encourage utilization similar to the Modified Checklist for Autism in Toddlers (M-CHAT; Robins et al., 2014). Specifically, it could be used in FXS clinics that often see patients back on a routine basis and use a multidisciplinary approach. Notably, since both the initial level and trajectory of change predicted elevated social anxiety over time, this measure could be utilized both at an individual appointment or across time. If used in both ways, analysis of results across time could allow for the potential identification of increased risk. In addition, this could be used as a target of a pharmaceutical or behavioral intervention trial to ameliorate anxiety symptoms in young children with FXS.

Contrary to our hypothesis, averted gaze and observed social behavioral inhibition were not significant predictors of later social anxiety. Despite previous research not finding a significant relationship between gaze behavior and an overall anxiety symptom measure (Roberts, Crawford, Will, et al., 2019), the authors thought this may be due to the evidence from the NT literature that suggests unique gaze profiles are associated with different types of anxiety (Waters et al., 2008, 2010), Specifically, previous research has identified a relationship between averted gaze and social anxiety in neurotypical research the authors hypothesized that an increased proportion of averted gaze would be related to social anxiety specifically. The lack of relationship has multiple potential explanations. First, social avoidant eye gaze is one of the most pervasive phenotypic features within

FXS emerging in infancy and persisting into adulthood (Hall et al., 2006,2009; Roberts, Crawford, Hogan, et al., 2019; Roberts, Crawford, Will, et al., 2019) Thus, we would expect to see increased gaze avoidance in the presence of a novel person that may not be distinctively related to social anxiety symptoms. In addition, social gaze avoidance within FXS could be an amalgamation of the increased risk of multiple comorbid diagnoses that are associated with atypical eye gaze. For example, Scherr and colleagues (2017) found differences in which young children with FXS averted their gaze dependent on ASD symptom severity during a novel social task, with lower levels of ASD symptom severity associated with a higher proportion of time looking towards their mother. This highlights that differing symptom severity of other prevalent comorbid conditions may influence the specific gaze patterns observed and may need a more nuanced analysis to identify relations between gaze and specific conditions.

Contrary to our hypotheses and existing literature, no cardiac indices were related to social anxiety symptoms at outcome. Baseline IBI at 24 months of age and the rate of change of IBI were related to overall anxiety symptoms in young children with FXS (Hogan et al., 2021). The current study did not find that the rate of change or initial level of baseline IBI was related to a specific social anxiety score. One notable difference is that the current study uses a social anxiety-specific measure, not a general anxiety measure. This may indicate that baseline IBI is related to generalized anxiety rather than social anxiety or other more discrete anxiety disorders. Moreover, previous research found no relation between baseline RSA and general anxiety symptoms but did find a relationship between baseline RSA symptoms and autism symptom severity (Hogan et al., 2021). This finding is inconsistent with our results which found no relationship

between baseline RSA and ASD symptom severity. However, Hogan and colleagues (2021) examined the baseline RSA prediction of later ASD symptom severity, whereas the current study compared ASD symptom severity and baseline RSA concurrently.

Consistent with neurovisceral integration theory, concurrent measurement of behavioral and cardiac markers of social anxiety were related in the current study (Beauchaine & Thayer, 2015). Specifically, the relationship between early cardiac and behavioral markers of social anxiety in the FXS group demonstrated that increased observed social behavioral inhibition was related to certain aspects of cardiac functioning during a novel social task in the FXS group. Specifically, observed social behavioral inhibition was related to IBI reactivity, not RSA reactivity, suggesting that behavior was more closely related to a measure of overall autonomic system functioning rather than just the parasympathetic system. Additionally, the relationship between IBI, not RSA, and social behavioral inhibition may suggest that the sympathetic branch of the ANS is driving the association between cardiac and behavioral indices. The sympathetic branch of the ANS is often thought to mediate the "fight or flight" response and is involved in fear responses exhibited by individuals in stressful social situations (Porges, 2007). Given that social behavioral inhibition is excessive fear in response to a novel person, we would expect increased sympathetic activity. Thus, the relationship between increased social behavioral inhibition and IBI may represent a "fight or flight" response with both physiological and behavioral responses attributed to the increased activation of the sympathetic nervous system activity. This is consistent with other research on FXS that does suggest that individuals with FXS have an overactive sympathetic nervous system and an underactive parasympathetic system (Baranek et al., 2008; Hall et al., 2009).

The current study found no relation between early cardiac markers and social anxiety symptom severity at outcome, which refutes current neurotypical literature. Neurovisceral integration theory posits that a flexible and adaptive ANS allows for increased prosocial behavioral and emotion regulation, which allows for an appropriate response in social situations. Atypical ANS response has been related to social anxiety symptoms in NT populations (Alkozei et al., 2015; Thayer & Lane, 2000), In FXS, ANS hyperarousal has been implicated as a hallmark feature (Cohen, 1995) and has been related to numerous phenotypic behaviors including social avoidance and general anxiety (Heilman et al., 2011; Hogan et al., 2021). Despite examining behaviors associated with social anxiety, no study has directly examined the relationship between early cardiac markers and later social anxiety. The lack of predictive utility of the cardiac markers on social anxiety symptoms may be related to ANS dysfunction that is related to numerous phenotypic features within FXS.

Strengths of the current study include the large size of the sample within this age range that is well characterized utilizing multi-method gold standard assessment tools. In addition, this is the first study using a longitudinal biobehavioral approach that includes multiple behavioral and physiological markers. This approach is critical, given the dynamic development within this age range and the phenotypic complexity within FXS. In addition, this is the first study to examine these early markers and their sensitivity to predict social anxiety symptom severity which is highly prevalent and impairing within this population.

This study is not without limitations. First, due to the availability of data and the difficulty of data collection within the sample, the sample age range for observed social

behavioral inhibition and eye gaze was limited. The use of a data-driven approach that only requires three out of the four behavioral markers may introduce potential challenges. Future studies should examine each construct separately to accurately model the development of these measures in isolation. In conducting separate analyses for each marker within the composite, it may highlight relationships that otherwise are not observed. In addition, the measurement of social anxiety outcome data utilized a measure not normed in FXS. Also, the measurement of social anxiety occurred at an age at which social anxiety symptoms typically are mild, which our data supports (Grant et al., 2005). Future studies should work on developing a specific social anxiety measurement tool within FXS or norm a current assessment tool within this population. Additionally, continued longitudinal follow-up should occur to examine social anxiety at older ages.

Overall, the current study has multiple important research and clinical practice implications. For research, this highlights the longitudinal trajectories of four cardiac indices and three early behavioral markers of social anxiety. Importantly, each of these demonstrated differing developmental trajectories that could emphasize the unique contributions of the different markers. In addition, it demonstrates a strong relationship between parent-reported behavioral inhibition and social anxiety symptoms. These findings, in turn, inform clinical practice, such that increased utilization of measures examining behavioral inhibition can be utilized to identify and potentially help treat social anxiety in FXS.

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