ANXIETY PROBLEMS AND STRUCTURAL AMYGDALA VOLUME DIFFERENCES IN AUTISTIC AND NON-AUTISTIC YOUTH

by

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Bachelor of Science
George Mason University, 2022

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DEDICATION

This manuscript is dedicated to my parents, my sister Megan, and especially to my closest collaborator: my cat, Noffy.

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I would like to thank the many friends, relatives, and colleagues whose unconditional support made this possible. I want to extend my appreciation and many thanks to Drs. Allison Jack, Goldie McQuaid, Jim Thompson, and Jane Flinn, in particular, for their guidance, feedback, support, and encouragement.

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LIST OF ABBREVIATIONS

ADI-R ..... Autism Diagnostic Interview -- Revised
ADOS-2 ..... Autism Diagnostic Observation Schedule, 2nd Edition
AFAB ..... Assigned Female at Birth
AMAB ..... Assigned Male at Birth
ASD ..... Autism / Autistic
ASDf ..... Autistic Females
ASDm ..... Autistic Males
CBCL……..Child Behavioral Checklist
DAS-2 ..... Differential Ability Scale, 2nd Edition
ICV……...Intracranial Volume
SRS-2 ..... Social Responsiveness Scale, 2nd Edition
TD……...Typically Developing
TDm……...Typically Developing Male
TDf…….Typically Developing Female

ABSTRACT

ANXIETY PROBLEMS AND STRUCTURAL AMYGDALA VOLUME DIFFERENCES IN AUTISTIC AND NON-AUTISTIC YOUTH

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George Mason University, 2023

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Autism is a cluster of highly heterogenous neurodevelopmental conditions that impacts differences in socio-emotional information processing; however, to date, little work has focused on the relevant brain systems involved in anxiety-related emotional processing in autistic youth. Importantly, despite recent efforts in broadening characterizations of the different presentations of autism based on sex assigned at birth, little is known about differences in psychopathological and neurodevelopmental trajectories between autistic girls and autistic boys. The present study interrogated neurodevelopmental differences in bilateral structural amygdala volumes in a sex balanced sample of autistic and non-autistic girls and boys aged 8-17 years old; we tested for effects of sex, IQ, diagnosis, along with anxiety problems and social behavior problems, in addition to interactions among these variables. Overall, between group comparisons did not reveal meaningful statistical differences in normalized left and right amygdala volumes when comparing autistic and typically developing (TD) counterparts. Autistic females (ASDf) reported significantly greater anxiety problems relative to typically developing females TDf. Additionally, autistic males (ASDm) and ASDf did not differ on anxiety or social

behavioral problem measures. As well, ASDf anxiety as a predictor of left or right amygdala volume did not survive statistical significance.

INTRODUCTION

Autism (henceforth: Autism Spectrum Disorder (ASD)) are highly heterogenous neurodevelopmental conditions, and involve differences in social information processing, along with the presence of restricted and repetitive behaviors and interests (APA, 2013). Differences in the brain structures relevant to anxiety and emotional processing (e.g., amygdala) have been frequently noted in ASD (Nordahl et al., 2020; Andrews et al., 2022; Lee et al., 2022; Herrington et al., 2017). Anxiety levels are strikingly elevated among the ASD population, with more than 40% of ASD individuals reporting the presence of at least one co-morbid clinically significant anxiety disorder (Herrington et al., 2017), as well as UK population-based data reporting the prevalence of an anxiety disorder to be as high as 50% among the ASD community (South et al., 2017). Autistic people report that co-morbid anxiety disorders frequently pervade social interactions and impact their daily living, often more so than ASD symptomatology itself (Kerns et al., 2014; van Steensel et al., 2011). The complex nature of anxiety combined with recurrent themes of social confusion and poor social execution (see also, for review: Volkmar & Wolf, 2013) commonly observed in ASD (Bellini, 2004) may appear to heighten difficulties in building friendships, and impact quality of life in daily interactions (van Steensel et al., 2011; Hennessy et al., 2022). As well, the biological underpinnings that regulate and promote anxiety-related disturbances in autistic youth and adolescence remain to be determined.

Identifying neurological substrates of anxiety traits in autism across development may be helpful to provide proper recognition and treatment of anxiety in these clinical populations. Critically, Females are underrepresented in ASD research and are four times less likely to receive an ASD diagnosis relative to ASD males (Loomes et al., 2017). However, research

suggests ASD females are diagnosed later than ASD males (Loomes et al., 2017. Autistic females may also appear to "camouflage" or hide their autistic traits more than their autistic male counterparts, and this is likely one aspect of why autistic individuals assigned female at birth are often misdiagnosed or undiagnosed (Lai et al., 2017) and underrepresented in research participation (Jack et al., 2021). Limited data suggests youth psychopathology to be, on average, higher in autistic girls versus autistic boys and typically developing (TD) girls (Nordahl et al., 2020).

Many parent-report anxiety trait measurements use scales such as Child Behavioral Checklist (CBCL) (Magyin & Pandolfi, 2017) and Spence Children's Anxiety Scale-Parent version (Glod et al., 2017). These measurements have frequently illustrated anxiety characteristics in autistic children that are consistent with *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.) anxiety disorders (often termed "common" anxieties) (Lau et al., 2020). However, other anxieties exclusive to autism may be categorically different from DSM-5 anxiety disorders, which is often classified as "autism-specific" anxieties in the literature. Anxieties specific to autism include social confusion, sensory overload etc. Differences in what causes anxiety for autistic individuals has yet to be classified in the DSM-5 (APA, 2013). For example, one characteristic of social phobia in the DSM-5 is described as "worry of negative evaluation from peers", whereas autistic individuals regularly demonstrate distress in social situations due to social confusion about neurotypical social semantics (see also, for review Volkmar & Wolf, 2013; Andrews et al., 2022). Furthermore, capturing anxiety in ASD youth relies heavily on parent-report responses, which remains challenging due to the complex nature of understanding how neurodivergent thinkers process social information. Historically, researchers have used CBCL anxious and depressed subscales to measure anxiety and have used

this (CBCL-AD) subscale to predict anxiety and neuroendophenotypes of anxiety in ASD youth (Juranek et al., 2006; see also, for review: Magyar & Pandolfi, 2017). **However, little to no research have instead employed the "CBCL anxiety problems" to characterize anxiety in this clinical population. This subscale separates anxiety from affective symptoms and may help us to pull apart anxious problems from depressive problems in autistic youth to develop actionable knowledge about the brain behavior relationship of neurobiological systems critical to emotional processing that will ultimately advance clinical treatment for autistic populations.** Ideally, however, future directions should aim to develop anxiety measurement scales created by autistic researchers to get at the subtly and complexity of ASD socio-emotional processing that neurotypical researchers often miss during evaluation (Ratto et al., 2022).

Anxiety is a major risk factor for poor mental health outcomes (Seguin et al., 2022) and is linked to reductions in quality of life (see also, for review: Olatunji et al., 2007). Some common anxiety inducing symptoms in autism can be described by sensory overload, specific phobias, and fear of change and unpredictability (Lau et al., 2020). Clinically significant anxiety, specific phobia, and anxiety disorders have been frequently examined in literature reviews and meta-analytic approaches interrogating anxiety in autistic populations, with findings of elevated anxiety in autistic youth to be 30-50% (van Steensel, Bogels, and Perrin, 2011; White et al., 2009) adults 40-50% (see also, for review: Kent & Siminoff, 2017. Comparatively, clinically significant anxiety has been reported to be prevalent in 3-5% of the general population (Salari et al., 2020). **The discrepancy of anxiety prevalence in autism compared with the general population is a pressing issue that demands more attention and investigation**. In addition,

tools used to capture and stratify “autistic-specific” anxiety and DSM-5 oriented anxiety developed from autistic individual’s perspectives are lacking.

The putative heightened levels of anxiety regularly seen in autistic versus the general population has sparked meaningful conversation about the relationship between ASD and anxiety (Kanner, 1943). Some autism researchers have suggested several theories that may help us better understand anxiety in ASD populations: some have suggested anxiety is a universal characteristic of ASD, or that anxiety is a distinct co-morbidity with ASD, or that the presence of an ASD itself alters how anxiety is manifested in the brain and in observable behavior (see also, for review: Wood & Gadow, 2010). **Nonetheless, whether anxiety is a feature of autism or is instead a highly co-occurring condition in autism, understanding the brain-based (e.g., differences in morphology) representations that promote anxiety in ASD is crucial to our ability to better understand and treat autistic persons with co-occurring anxiety.**

Amygdala and Anxiety in ASD

The amygdala has long been known to be a hub crucial for processing emotional information, and to activate in response to perceived danger in the environment (see also, for review: DeCampo & Fudge, 2012) . The amygdala is a small bilateral structure based deep in the medial temporal lobes and has been implicated in various circuits important for social information processing, emotional regulation, and anxiety-related disturbances (Seguin et al., 2022). Neurodevelopment of amygdala throughout childhood is highly variable, with most reports highlighting a critical window of accelerated growth of this structure seen in early post-natal development (Uematsu et al., 2012). Remarkably, TD youth with especially large amygdala volumes in early post-natal life have been noted to have higher incidence of a later diagnosis of a neurodevelopmental condition such as ASD (Avino, 2018; Shen et al., 2022). Amygdala volume

may therefore be an important biomarker for identifying ASD early-on, particularly for populations most at-risk of going undiagnosed or misdiagnosed, such as individuals assigned female at birth (Shen et al., 2022; Jack et al., 2021).

Longitudinal work has illuminated a morphological trajectory of the amygdala in a mildly sex-balanced ASD sample, whereby accelerated growth is observed in early childhood, followed by deceleration—and null brain differences of amygdala volume by adolescence (Schumann et al., 2004, 2010; Schumann & Nordahl, 2011). However, this framework has been difficult to replicate, largely due to mixed findings in individual differences in amygdala volume in ASD samples and different methodological approaches. Extant literature on morphological differences seen in amygdalar volume based on distinct anxiety disorder subtypes in ASD is lacking.

Several studies have investigated amygdala volume in non-autistic anxious youth and adult populations. Most of these interrogations have pointed towards decreased amygdala volume as a biomarker for an anxiety disorder (Blackmon et al., 2011; Hayano et al., 2009; Mueller et al., 2013). One suggested hypothesis that might partly explain increased atrophy of amygdalar volume observed in individuals with an anxiety disorder is that anxiety induced hyperactivation of amygdala firing over a prolonged period can lead to excitotoxicity; moreover, overactive firing of the amygdala over an extended time can activate a surplus of glutamatergic receptors at the synapse which leads to decreased firing of neuronal activity in the amygdala that ultimately orchestrates cell death (Blackmon et al., 2011). Contrarily, some studies have suggested enlarged growth—and not decreased volume—of the amygdala to be reflective of an anxiety disorder (Quin et al., 2014). Alterations or increases in amygdala volume might be explained to a limited extent by reductions in pruning during adolescence in ASD (Quin et al., 2014). However, more empirical work is necessary to elucidate the mechanisms and

morphological differences seen in amygdala volume in ASD youth, and how anxiety and might be connected to alterations in amygdala morphometry.

Amygdalar Subnuclei

The amygdala is made up of at least 13 sub nuclei (see also, for review: Ressler, 2010), and most recently has been stratified by 18 subnuclei (9 on each side) using high resolution MRI images from a segmentation algorithm (Saygin, 2017). The most prominent of these subnuclei are the central nuclei (CN), Basal nuclei (BA), and lateral nuclei (LN) (see also, for review: Ressler, 2011). CN is important for conditioning a fear response and regulating cortisol through circuitry with paraventricular nucleus of the hypothalamus (Davis, 1992). BA and LA have historically been known to be important for associative learning and fear conditioning; such that, LA receives auditory and visual sensory input, while BA is involved in further processing LA input, while pairing conditioned and unconditioned stimuli (CS-US) to be sent to CN to consolidate learning (Janak, 2015).

In addition to being implicated in anxiety, studies have demonstrated the involvement of amygdala in socio-emotional processing (Nordahl et al., 2020; Yarger, Nordahl & Redcay, 2022; Andrews et al., 2022; Lee et al., 2022). Amygdala function is essential for recognizing fearful faces and detecting for salience of stimuli presented in the environment (Herrington et al., 2017). A systematic review looked at 55 functional magnetic resonance imaging studies and illuminated 25 of the studies to show amygdala activation during a fearful face task paradigm, while only 4 studies showed amygdala activation in response to positive feedback (see also, for review: Phan et al., 2002). In autism, research has revealed amygdala enlargement witnessed in early childhood is associated with elevated social and communicative differences (Lucibello et al., 2019; Munson et al., 2006)

Although the amygdala has widespread implications in regulating socio-emotional processing (Truitt et al., 2007), many researchers have looked at structural volume of the amygdala across development in ASD (Nordahl et al., 2012; 2020; Li et al., 2019). Emerging evidence has illuminated a pattern of enlarged amygdala volume in early post-natal life (Schumann et al., 2009; Li et al., 2019) to be associated with ASD individuals who received a late diagnosis (Avino et al., 2018). Remarkably, early post-natal overgrowth of amygdala can be detected in infants young as 6 months old (Li et al., 2019). Many structural neuroimaging studies have suggested there exists a critical window in autistic samples for detection of overgrowth in amygdala volume during early childhood (Li et al., 2019). **Therefore, spotting accelerated amygdala volume in at-risk youth for autism may be exclusive to infants in early post-natal neurodevelopment (Shen, 2022), and this sensitive time period of collecting brain images in youth might help us better identify ASD individuals, especially those who are at high risk of going misdiagnosed or undiagnosed.**

Anxiety and ASD

Anxiety is a complex component of social behavior and can be measured in several ways. Typically, anxiety levels in ASD youth are collected via parent-reports (Child Behavior Checklist), self-reports, or clinicians conducting clinical interviews with participants/patients (Magyar & Pandolfi, 2017; Hennessy et al., 2022). The few studies that have investigated structural amygdala volume as a predictor of anxiety levels have led to mixed findings, likely due to different methodological approaches in measuring anxiety. Increased amygdala volumes have been linked to anxiousness in autistic youth via parent-reports of child anxiety levels (Juranek et al., 2006). Conversely, ASD children with high parent-reported anxiety displayed, on

average, smaller right amygdala volume relative to controls (Herrington et al., 2017). Recent work reported that autistic and typically-developing youth and early adolescents do not demonstrate differences in amygdala volume based on anxiety levels (Yarger et al., 2021). Alterations and differences in amygdala volume have also been implicated non-autistic populations, with adolescents and adults with clinically significant anxiety disorders reporting atypical amygdala volumes (De-Billis et al., 2000). Additionally, differences in amygdala volume have been reported in attention deficit hyperactivity disorder (ADHD) and obsessive compulsive disorder (OCD) (Baribeau et al., 2019; Seguin et al., 2021). Given the high overlap of shared traits documented in ASD, ADHD, and OCD, it has been hypothesized that a connection between amygdala volume and ASD severity would be apparent in ASD youth and ASD adolescence (Seguin et al., 2021).

Amygdala Subnuclei and Functionality

As mentioned previously, CN, BA, and LA subnuclei, also known as "deep nuclei", have been well documented to be important for socio-emotional processing, emotional regulation, fear detection, autonomic function, and prediction based on incoming sensory stimuli (Truitt et al., 2007). Notably, BA and LA are intrinsically linked to the hypothalamic-pituitary adrenal (HPA) feedback loop, and LA has been suggested to relay stress signals via cortisol to this HPA axis (Herman, 2016). Additionally, BA and LA comprise of the basolateral complex (BLA), which projects onto cerebral regions such as the orbitofrontal cortex (OFC) and pre-frontal cortex (PFC). Importantly, BLA has been noted to be involved in predicting and executing social responses to environmental and social stimuli (Truitt et al., 2007; Hennessey et al., 2022). In animal models, BLA activation to medial pre-frontal cortex has been proven to be reflective of

heightened anxiety, while decreased activation of this circuit has been shown to exemplify decreased anxiety (Felix-Ortiz et al., 2016). A recent report suggested that enlarged BA and LA volumes in ASD samples may be predictive of barriers to social execution (Seguin et al., 2021). **As well, alterations in BA and LA activation have been posited to underpin differences seen in ASD individuals in responding to social ques in a timely and nimble fashion** (Nacewicz et al., 2006; Sinha et al., 2015).

In addition to the previously stated "deep nuclei" sub regions of the amygdala, there is also present several bundles of subnuclei within the amygdala that have important involvement in mechanisms crucial to behavior and cognition. Notably, paralaminar nucleus (PL) is a subregion located deep in the amygdala and overlaps many deep nuclei such as BA and LA. Some studies have suggested PL is an extension of the BA nucleus (Barbas and de Olmos, 1990; see also, for review: deCampos & Fudge, 2013), while other studies have claimed PL to be separate nucleus and has been identified under several different names, including "amygdaleum profundum ventral" (Brockhaus, 1938), "granular nucleus" (Braak & Braak, 1983), before ultimately being coined as "paralaminar nucleus" (Amaral & Price, 1984; De Olmos, 2004; see also, for review: De Campo & Fudge, 2012). Although there is no clear boundary separating PL from BA and LA, PL appears to be more abundant in glial cells, including interneuron cells that morphologically resemble glial cells (Ramon y Cajal, 1909; Kuwaguchi et al., 1997), which makes cellular architecture of PL highly variable (Amaral et al., 2002; Braak & Braak, 1983).

Heterogenous cellular composition and lack of agreeability over the nomenclature of PL, along with robust differences seen in size and function across species, has thus made this subregion difficult to study. However, human PL has been implicated in psychiatric disorders

such as schizophrenia and anxiety disorders (Seguin et al., 2021; Hennessey et al., 2022), and may be an important biomarker in identifying anxiety in at-risk youth.

Central nucleus (CN) of the amygdala has long been regarded as the main efferent pathway of the amygdala and is thought to strongly influence regulation and response to threatening stimuli (LeDoux et al., 1990). CN also controls and releases hormones important for mood regulation, such as cortico-releasing factor (CRF), which projects onto the anterior pituitary gland in the hypothalamus and is released from the adrenal cortex to the central nervous system (CNS) and peripheral nervous system (PNS) in response to stress (Vale, 1981). CRF is linked to the HPA-axis, which is crucial to our ability to maintain mood and execution of behavior (see also, for review: Bao & Swaab, 2019).

A recent study found BLA volume to be larger in ASD adolescents relative to TD controls, and BLA and CN growth to be correlated with restrictive and repetitive behaviors and interest (RRBI) scores using the ADI-R scale (Seguin et al., 2021). It is important to note that RRBI is commensurate with anxiety seen in ASD (Jiujias et al., 2017; Halls et al., 2015).

To our knowledge, only one extant study has looked at amygdala volume in relation to CBCL anxious/depressed (CBCL-AD) scores in ASD youth (Juranek et al., 2006). Results concluded total amygdala volume and right amygdala volume to be predictive of CBCL-AD scores. However, the inferences drawn from these results yielded several flaws: only 7 of the 42 ASD participants were assigned female at birth; the CBCL-AD summarized 14 anxious and depression measurements as the main predictor of anxiety and amygdala volume. This study did not use DSM-5 specific oriented anxiety using CBCL anxiety problems and CBCL affective problems subscales as a predictor of amygdala volume. Furthermore, recruiting a sex balanced sample of ASD youth and measuring DSM-5 anxiety specific traits as described in the CBCL

anxiety problems and CBCL affective problems better assess and analyze clinically significant anxiety in ASD youth has yet to be interrogated.

Given the variety of measurements used to assess anxiety in ASD populations (e.g. self-report, parent-report, clinical interview), and diverse nature of different anxieties' neural mechanisms and observable presentations throughout the lifespan, it is difficult to capture anxiety using one metric. Although self-reports of anxiety in ASD youth may allow for keen insight into internalizing behaviors, parent reports are also necessary, as they aim to capture additional anxiety symptoms that ASD youth may lack the ability to self-identify. Parent report measures also allow the parent perspective of behavioral changes seen in their child throughout child development.

HYPOTHESES

Little work has examined anxiety in ASD youth using a sex-balanced sample (Nordahl et al., 2020; Andrews et al., 2022; Lee et al., 2022). Recent evidence emerged, suggesting ASD female adolescents compared with their ASD male counterparts, experience elevated occurrence of emotional dysregulation (Kreiser et al., 2015) along with increases in internalizing behaviors (Solomon et al., 2012; Nordahl et al., 2020). Autistic individuals, on average, report more anxiety-related disturbances relative to their TD counterparts. Amygdala volume has been widely implicated in autism and anxiety. Enlarged amygdala volume in autistic individuals has also been hypothesized to follow a trajectory of accelerated growth in early post-natal life—followed by decreased growth in late childhood—and null brain differences by late adolescence (Herrington et al., 2017; Nordahl et al., 2020). No studies, however, have examined amygdala volume in relation to anxiety in autism, or have stratified amygdala volume associations to distinct co-morbid anxiety and affective disorders in autism as described by the CBCL anxiety and affective problems subscale.

The present study therefore aims to disentangle anxiety disturbances from depression and delineate its potential amygdala associations in a unique sex-balanced sample of ASD youth and TD youth. To measure anxiety and depression, we will use two *DSM-5*-oriented CBCL subscales: anxiety problems and affective problems.

Behavior: We posit autistic females (ASDf) will report higher anxiety compared with autistic male (ASDm) counterparts and typically developing females (TDf). We

additionally hypothesize ASDf will report greater social behavior problems relative to ASDm.

Brain: We hypothesize ASD youth compared with TD controls will report larger left and right amygdala volumes.

Brain-behavior correlations: We predict a positive association of amygdala volume with CBCL anxiety subscale in ASDf, and positive association of amygdala volume with CBCL affective problems subscale in ASDf.

METHODS

The present study used data collected from Wave 1 of the GENDAAR project (National Database for Autism Research Data Collection #2021). Sample data was gathered across four sites in the United States (Yale University, Seattle Children's Hospital/University of Washington, Boston Children's Hospital/Harvard Medical School, and the University of California Los Angeles). This sample consists of autistic and typically developing youth aged 8-17 years, with a full scale IQ >70 as estimated by the Differential Abilities Scale (Second Edition) (Eliott, 1990) General Conceptual Ability Standard Score. Exclusion criteria for every participant included the following: any known genomic condition (e.g., Fragile X syndrome, Down's syndrome), neuropathological disorders that occur above the brainstem (except for uncomplicated non-focal seizures), any seizure incidence in the last year, and inability to comply and/or comprehend instructions involving a task.

Diagnostic status for participants was measured and confirmed by expert clinicians using the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) (Lord et al., 2012) and the Autism Diagnostic Interview Revised (ADI-R) (Lord et al., 1994). Participants who met either ADOS or ADI-R criteria were included in the autistic sample. Inter-site reliability was maintained for these diagnostic measures by having expert clinicians re-code one ADOS-2 and one ADI-R bi-annually (every 6 months), with further maintenance held of these measures by having the lead clinician at each scan site double code 10% of all assessments conducted.

TD were included only if they did not have first-or-second degree relative(s) with autism spectrum, developmental, neurological, or psychiatric condition(s). Further inclusion criteria for TD participants required them to exhibit no evidence of elevated (total t-score ≥ 65) autistic traits

using the Social Responsiveness Scale 2nd Edition (SRS-2) parent report (Constantino & Gruber, 2012) .

For inclusion in analyses for the current report, participants were required to have a MRI anatomical scan of adequate quality (see below for quality assurance procedures), a complete IQ assessment and a valid Child Behavioral Checklist (CBCL; Maygin & Pandolfi, 2017) anxiety subscale score. Participants were additionally excluded from these analyses if they had a biological sibling in the same study. These inclusion/exclusion steps were conducted before data analysis were performed. 257 children met inclusion criteria based on MRI data quality. Groups were matched for age, full scale IQ, and sex, resulting in a final sample of $N = 231$ youth ($n = 59$ ASDf*;* $n = 63$ ASDm*;* $n = 56$ TDf*;* $n = 53$ TDm).

Participants were asked to complete a structural MRI scan across four sites: Harvard Medical School, Seattle Children's Hospital, UCLA Medical Center, and Yale Medical Center. Given the age range of our sample (8-17 years old), trainees who conducted the MRI scans received extensive training in preparing youth for an MRI scan. The additional measures carried out to ensure minimal head motion noise involved placing participants in a mock MRI scanner before running participants in the scanner. To minimize head movement during the scan, desensitization techniques were implemented prior to the participants' imaging visit. These techniques included the use of social stories to explain what would happen during the imaging visit. Parents were provided with instructions to help prepare their child for the imaging visit, including receiving audio and video clips to help participants prepare for the scan environment. Some [participants also underwent a mock scan during which the scan environment was simulated, including audio of scan sequences.

To minimize head motion during the actual scan, foam head stabilizers were wedged into the head coil. Participants who found it comforting were wrapped in a sheet or blanket. This wrapping both provided comfort to the participant while reminding them not to move while in the scanner. Participants authorized written informed consent (parent) and assent (child), and all research activities were in good standing with each university's Institutional Review Board and the Declaration of Helsinki.

Quality assurance of raw MRI scans

Before processing via FreeSurfer, raw MRI scans were rated by a team of trained research assistants. In detail, trainees visually assessed scans and rated for motion-related noise and other artifacts, such as ringing and phase wrapping. To ensure standardization across raters, training included rating a set of 20 scans that was assigned to all raters. After training on these 20 scans, raters were assigned batches of unprocessed MRI scans. Each scan was rated by at least two independent raters. Scans were rated on a scale from 0 to 2 as follows: 0 = "unusable scan," 1= "usable but not high quality" and 2 = "usable." "Unusable scans" designated those scans with severe motion and/or other artifacts that made them unsuitable for use in FreeSurfer. Scans rated as "usable but not high quality" were scans that showed, for example, some evidence of motion artifacts but not severe artifacts that would render them unusable for processing and analyses. Differences between ratings and scans rated as "1" (i.e., "usable but not high quality") were discussed during group meetings to determine the final set of scans submitted to FreeSurfer.

Data Analysis and Hypotheses

This study sought to disentangle anxiety from other common psychiatric conditions and delineate its potential associations with amygdala structure in a unique sex-balanced sample of ASD youth and TD youth. We measured anxiety in both autistic and TD samples, by collecting parent report on the *DSM-5*-oriented CBCL anxiety problems subscale. We used these scores as predictors of intracranial volume (ICV)-normalized left and right amygdala volumes in our ASD samples.

We used a general linear regression model (GLM) for both behavioral and brain analyses. CBCL anxiety problems was used in our bivariate regression model of ASDf predicting left and right amygda volume. The proportional method of dividing right and left amygdala volumes by ICV volume sought to address variance in brain volume related to age and sex. The ICV-normalized ratio was used for analyses of amgydala volume.

Descriptive Statistics

Descriptive statistics are provided for all variables of interest (Table 1.1). All within and between diagnostic group comparisons were analyzed using Welch's independent samples t-tests. Between-group comparisons for ASD versus TD on all relevant variables can be found on Table 1.1.

Objective 1.

To examine the differences of CBCL anxiety problems scores by sex within the ASD sample and between ASDf and TDf. To address this objective, Welch's independent-samples t-tests were conducted comparing ASDf and ASDm, and ASDf and TDf. This allowed for gender-normed comparison of ASDm and ASDf, as well as between group differences by sex between

ASDf and TDf. Means of the ASD group were centered when analysis was compared with means of the TDf group.

Objective 2.

To assess differences of left and right amygdala volumes between ASD and TD samples. To address this objective, Welch's independent-samples t-tests were conducted to test mean differences of left and right amygdala volume (ICV – normalized) when comparing the ASD and TD samples. Proportional normalization of left and/or right amygdala volumes were conducted as a ratio of left and/or right amygdala volumes.

Objective 3.

To assess the relationship between ASDf CBCL anxiety problems and normalized right and left amygdala volumes. We used a bivariate regression model to address this objective, using the following equations:

Bivariate regression model 1: Right amygdala volume (ICV-normalized) = ASDf CBCL anxiety problems.

Bivariate regression model 2: Left amygdala volume (ICV-normalized) = ASDf CBCL anxiety problems.

Neuroimaging Processing Data

Manual segmentation is considered a gold-standard in the delineation of subcortical structures, particularly in clinical contexts; however, with large datasets in research contexts, automated methods, such as FreeSurfer, are necessary. FreeSurfer provides an automated

segmentation of the amygdala that have been shown to be moderately correlated with manual segmentation (Grimm et al., 2015).

Left and right amygdala volumes were processed automatically using FreeSurfer (http://surfer.nmr.mgh.harvard.edu/) version 6.0. This automated segmentation algorithm is based on Bayesian inference, and the amygdala atlas reflects (n = 10) ex vivo human temporal lobes (Fischl, 2002). Other automated methods of segmentation of subcortical structures including the amygdala, such as voxel-based morphometry (VBM) have been shown to yield largely comparable volume estimations with FreeSurfer, although some evidence suggests that VBM may demonstrate higher agreement with volumes extracted from manually specified segmentations compared to FreeSurfer (Grimm et al., 2015).

Further insight into anxiety-specific problems, paired with examination of left and right amygdala volumes (ICV – normalized) during child development in ASD and TD youth may help us better characterize: 1) pediatric anxiety disorders in ASD; and 2) morphological differences in lateralized amygdala volumes in relation to anxiety-specific differences in ASD.

BEHAVIORAL MEASURES

Child Behavioral Checklist (CBCL) – Anxiety Problems

CBCL anxiety problems reports were collected as a measurement of anxiety related disturbances as described by parents of each participant. The CBCL contains two empirically-derived (i.e., through factor analysis) broadband scales representing internalizing and externalizing problems, eight empirically-derived syndrome scales representing different patterns of co-occurring emotional and behavioral problems, and six DSM-Oriented scales derived through expert consensus (Magyar & Pandolfi, 2017). CBCL's Affective Problems and Anxiety Problems DSM Oriented Scales was completed and validated on 93 autistic youth, ages 6 to 18 years (*see also: Magyar & Pandolfi, 2017).*

The Differential Abilities Scale, Second Edition (DAS-2)

The DAS-2 was used to examine general cognitive abilities and intelligence. More specifically, the General Conceptual Ability Standard Score was used to estimate full-scale IQ (FSIQ).

The Social Responsiveness, Second Edition (SRS-2)

The SRS-2 (Constantino & Gruber, 2012) was gathered as a measure of autistic characteristics. The SRS-2 consists of five subscales: Social Awareness; Social Cognition; Social Communication; Social Motivation; and Restricted Interests and Repetitive Behavior. We used the SRS-2 to evaluate social behavior problems to address part of objective 2: to evaluate whether there were differences in SRS-2 score between ASDf and ASDm.

The Autism Diagnostic Observation Scale, Second Edition (ADOS-2)

The ADOS-2 (Lord et al., 2012) was administered by expert clinicians to confirm diagnoses of ASD. The assessing clinician used either Module 3 or Module 4, as appropriate based on clinical judgment.

The Autism Diagnostic Interview – Revised (ADI-R)

The ADI-R (Lord, Rutter, & Le Couteur, 1994) was also used to confirm autism diagnosis. ASD Participants were included if they met either ADOS-2 or ADI-R diagnostic threshold for ASD.

Neuroimaging Data

Neuroimaging data was collected on either a Siemens 3T Tim Trio scanner with a 12-channel head-coil, or a Siemens 3T Prisma Fit with a 20-channel head coil. An upgrade at two sites (Seattle Children's Hospital and UCLA) during the course of data collection led to these differences. The anatomical image used in these analyses was a whole-brain T1-weighted anatomical image (Siemens MPRAGE – magnetization-prepared rapid gradient-echo sequence).

RESULTS

Descriptive Statistics

The means of each variable reported in each group can be located on Table 1.1 Mean comparisons were all conducted via *Welch's two-sample T-tests* using R programming software. Pearson correlations coefficients were additionally run in both bivariate regression models (Table 2.1; Table 2.2).

Sex and diagnostic group differences in anxiety

CBCL anxiety problems were significantly greater in ASDf relative to TDf t(df) = 8.27(66.20), $p < 0.001$; Table 1.2. Additionally, CBCL anxiety problems did not reveal differences between ASDf and ASDm t(df) = -0.35(114.14), $p = 0.7$; Table 1.3. Along with this, we did not find differences in social behavior problems (SRS-2) between ASDf and ASDm t(df) = -0.13(116.15*), p* = 0.09; Table 1.3.

Brain-based diagnostic group differences

When comparing left amygdala volume (ICV – normalized) between ASD and TD groups, no differences were found t(df) = -0.84 (218.53*), p* = 0.35; Table 1.1. Similarly, ASD and TD groups did not differ in right amygdala volumes (ICV – normalized) t(df) = -0.85 (227.0*), p* = 0.34; Table 1.1.

Brain-behavior correlations of ASDf

Contrary to our predictions, the overall bivariate regression model of ASDf predicting ICV-normalized right amygdala volume from CBCL anxiety was not significant (F(1, 56) = -1.17, P = 0.24, Adj R2 = 0.006), and the CBCL anxiety term was not significant (Table 2.1, Figure 1.1). Likewise, our overall model predicting ICV-normalized left amygdala volume from

ASDf CBCL was not significant ($F(1, 56) = 0.004$, $p = 0.95$, Adj $R2 = -0.02$), nor was the CBCL anxiety term within this model (Table 2.2; Figure 1.2).

All between-group differences for ASDf and ASDm can be found in Table 1.2. All within-ASD sex differences comparisons can additionally be found in Table 1.3. Our bivariate regression model descriptive statistics of ASDf CBCL anxiety predicting right amygdala volume (ICV-normalized) can be found on Table 2.1. Our bivariate regression model descriptive statistics of ASDf CBCL anxiety predicting left amygdala volume (ICV – amygdala) can be found on Table 2.2. Our bivariate regression model of ASDf CBCL anxiety predicting right amygdala volume (ICV – normalized) can be located on Figure 2.1. Lastly, Our bivariate regression model of ASDf CBCL anxiety predicting left amygdala (ICV – normalized) can be found on Figure 2.2.

Table 1.1: Matched Sample: Descriptive Statistics

Group	Autistic Female	Autistic Male	TD Female	TD Male	*df*	*t*-value	*p*-values (Between ASD and TD)
N	59	63	56	53			
Age (months)	157.23 (13.1 years; SD = 31.56 months)	154.06 (12.8 years; SD = 31.16 months)	159.24 (13.3 years; SD = 36.85 months)	161.42 (13.5 years; SD = 34.36 months)	221.10	-1.20	$p = 0.24$
CBCL anxiety	62.09 (SD = 9.36)	62.84 (SD = 8.37)	51.82 (SD = 3.54)	52.1 (SD = 4.03)	156.40	12.2	$p < 0.001$*
SRS-2 (raw)	92.90 (SD = 28.15)	92.92 (SD =26.82)	18.18 (SD = 12.1)	19.17 (SD = 17.31)	178.84	26.44	$p < 0.001$*
L Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD = 0.01)	0.11 (SD =0.01)	0.11 (SD =0.01)	218.53	-0.84	$p = 0.35$
R Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD =0.01)	0.11 (SD = 0.11)	0.12 (SD = 0.01)	227.0	-0.85	$p = 0.34$
IQ	102.28 (SD = 20.7)	101.38 (SD = 20.67)	112.68 (SD = 15.12)	113.22 (SD = 15.65)	218.80	-4.9	$p = 0.08$

Note: all data is summed into Mean scores and standard deviation scores (SD) as indicators of range. *df* =Degrees of Freedom. ICV = Intracranial Volume. TD = Typically Developing. ASD = Autism Spectrum Disorder. CBCL = Child Behavioral Checklist. SRS-2 = Social Responsibility Scales, *second edition.* L amygdala = Left amygdala. R amygdala = Right amygdala. IQ = Intelligence Quotient (as described by General Conceptual Ability Standard Score using the differential abilities scale, *second edition* (DAS-2).

Overall test, $t = -1.20$, $df = 221.10$, $p = .24$. Welch's two-sample T-test did not reveal differences in age between Autistic and TD groups.

Overall test, $t = 12.20$, $df = 156.40$, $p < 0.001$. Welch's two sample T-test reported significant differences in CBCL anxiety between Autistic and TD groups.

Overall test, $t = 26.44$, $df = 218.53$, $p < 0.001$. Welch's two sample T-test revealed significant differences in SRS-2 scores between Autistic and TD groups

Overall test, $t = -0.84$, $df = 178.84$, $p = 0.35$. Welch's two sample T-test did not reveal significant differences in normalized left amygdala volume between groups

Overall test, $t = -0.85$, $df = 227$, $p = 0.34$. Welch's two sample T-test did not reveal significant differences in normalized right amygdala volume between Autistic and TD groups

Overall test, $t = -0.85$, $df = 218.80$, $p = 0.08$. Welch's sample T-test revealed TD had greater average IQ than ASD participants just above the *a priori* $p < 0.05$ alpha level.

Table 1.2: Autistic and Non-Autistic Female Comparisons

Group	ASDf	TDf	*df*	*t*-values	*p*-values
N	59	56			
Age	157.23 (13.1 years; SD = 31.56 months)	159.24 (13.3 years; SD = 36.85 months)	104.50	-0.33	$p = 0.74$
CBCL anxiety	62.09 (SD = 9.36)	51.82 (SD = 3.54)	66.20	8.27	$p < 0.001$*
SRS-2 (raw)	92.90 (SD = 28.15)	18.18 (SD = 12.1)	79.16	18.40	$p < 0.001$*
L Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD =0.01)	97.35	-0.31	$p = 0.75$
R Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD = 0.11)	108.66	-0.03	$p = 0.97$
IQ	102.28 (SD = 20.7)	112.68 (SD = 15.12)	104.10	-3.2	p <0.001*

Note: All scores are summed into mean scores and standard deviations (SD) as range indicators. *df* = Degrees of Freedom. ASDf = Autistic Female. ICV = Intracranial Volume. TDf = Typically Developing Female. CBCL = Child Behavioral Checklist. SRS-2 = Social Responsibility Scales, *second edition.* L amygdala = Left amygdala. R amygdala = Right amygdala. IQ = Intelligence Quotient (as described by General Conceptual Ability Standard Score using the differential abilities scale, *second edition* (DAS-2).

Overall test, $t = -0.33$, $df = 104.50$, $p = 0.74$. Welch's two sample T-test did not reveal significant differences between age among ASDf and TDf.

Overall test, $t = 8.27$, $df = 66.20$, $p < 0.001$. Welch's two sample T-test revealed ASDf reported significantly greater CBCL anxiety relative to TDf.

Overall test, $t = 18.40$, $df = 79.16$, $p < 0.001$. Welch's two sample T-test revealed ASDf reported significantly greater SRS-2 problems than TDf.

Overall test, $t = -0.31$, $df = 97.35$, $p = 0.75$. Welch's two sample T-test did not reveal difference in normalized left amygdala volumes between ASDf and TDf.

Overall test, $t = -0.03$, $df = 108.66$, $p = 0.92$. Welch's two sample T-test did not reveal difference in normalized right amygdala volumes between ASDf and TDf.

Overall test, $t = -3.2$, $df = 104.10$, $p < 0.001$. Welch's two sample T-test revealed TDf had significantly greater average IQ relative to ASDf.

Table 1.3: Within ASD Sex Differences Comparisons

Group	ASDf	ASDm	*df*	*t*-values	*p*-values
N	59	63			
Age (months)	157.23 (13.1 years; SD = 31.56 months)	154.06 (12.8 years; SD = 31.16 months)	117.90	0.66	$p = 0.51$
CBCL anxiety	62.09 (SD = 9.36)	62.84 (SD = 8.37)	114.14	-0.35	$p = 0.7$
SRS-2 (raw)	92.90 (SD = 28.15)	92.92 (SD =26.82)	116.15	-0.13	$p = 0.9$
L Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD = 0.01)	118.00	-0.50	$p = 0.62$
R Amygdala (% ICV)	0.11 (SD =0.01)	0.11 (SD =0.01)	117.62	0.37	$p = 0.72$
IQ	102.28 (SD = 20.7)	101.38 (SD = 20.67)	117.50	0.17	$p = 0.86$

Note: All scores are summed into mean scores and standard deviations as range indicators. *df* = Degrees of Freedom. ICV = Intracranial Volume. ASD = Autism Spectrum Disorder. ASDf = Autistic Female. ASDm = Autistic Male. CBCL = Child Behavioral Checklist. SRS-2 = Social Responsibility Scales, *second edition.* L amygdala = Left amygdala. R amygdala = Right amygdala. IQ = Intelligence Quotient (as described by General Conceptual Ability Standard Score using the differential abilities scale, *second edition* (DAS-2).

Overall test, $t = 0.66$, $df = 117.90$. $p = 0.51$. Welch's sample T-test did not reveal significant differences between age among ASDf and ASDm .

Overall test, $t = -0.35$, $df = 114.14$, $p = 0.7$. Welch's two sample T-test did not reveal differences in CBCL anxiety among ASDf and ASDm.

Overall test, $t = -0.13$, $df = 116.15$, $p = 0.9$. Welch's two sample T-test did not reveal differences in SRS-2 reporting among ASDf and ASDm.

Overall test, $t = -0.50$, $df = 118.0$, $p = 0.62$. Welch's two sample T-test did not reveal differences in normalized left amygdala volume between ASDf and ASDm.

Overall test, $t = 0.37$, $df = 117.62$, $p = 0.72$. Welch's two sample T-test did not reveal differences in normalized right amygdala volumes between ASDf and ASDm.

Overall test, $t = 0.17$, $df = 117.50$, $p = 0.86$. Welch's sample T-test did not reveal differences in IQ among ASDm and ASDf.

Table 2.1: Bivariate Regression Model of ASDf CBCL Anxiety Predicting ICV-Normalized Right Amygdala Volume

	B	*SE*	*t*	*p*
Intercept	0.49	0.62	11.28	$P < 0.001$
CBCL Anxiety	-0.01	0.03	-1.165	0.25

Note: B = Coefficient of Determination. *SE* = Standard Error of the Mean. CBCL = Child Behavioral Checklist. ICV = Intracranial Volume. ASDf = Autistic Female. *t* = t-value. *p* = p value.

Overall bivariate regression test: Intercept of CBCL anxiety (B(SE) = 0.49(0.62), $p < 0.001$).

Overall bivariate regression test: The main effect of CBCL anxiety (B(SE) = -0.01(0.03), $p = 0.25$) was not significant in predicting normalized right amygdala volume.

Table 2.2: Bivariate Regression Model of ASDf CBCL Anxiety Predicting ICV-Normalized Left Amygdala Volume

	B	*SE*	*t*	*p*
Intercept CBCL Anxiety	0.76	0.55	9.44	*P* < 0.001
CBCL Anxiety	0.35	0.02	0.063	0.95

Note: B = Coefficient of Determination. *SE* = Standard Error of the Mean. Adj R Squared = Adjusted R Squared. CBCL = Child Behavioral Checklist. ICV = Intracranial Volume. ASDf = Autistic Female. *t* = t-value. *p* = p value.

Overall bivariate regression test: Intercept of CBCL anxiety (B(SE) = 0.76(0.55*), p* < 0.001).

Overall bivariate regression test: The main effect of CBCL anxiety (B(SE) = 0.35(0.02*), p* = 0.95) was not significant in predicting ICV-normalized left amygdala volume.

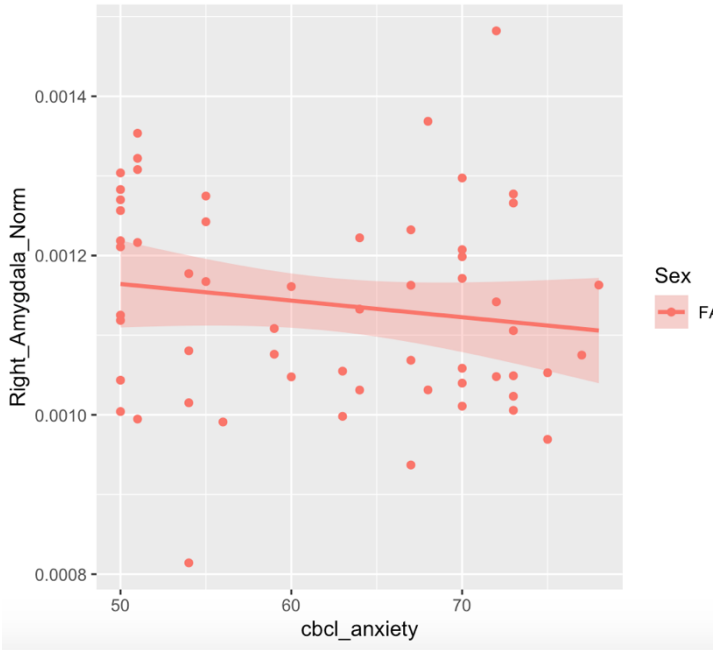

Figure 1.1: ASDf CBCL Anxiety Predicting ICV-Normalized Right Amygdala Volumes

Note: F = Autistic Female. ASDf = Autistic Female. CBCL = Child Behavioral Checklist. ICV = Intracranial Volume.

Overall bivariate regression test: The main effect of CBCL anxiety (B(SE) = -0.01(0.03*), p* = 0.24) was not significant in predicting normalized right amygdala volume

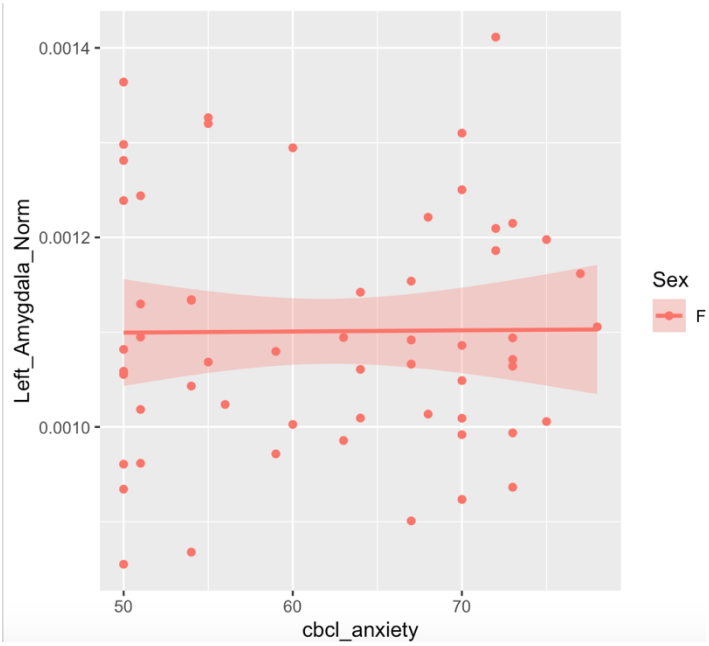

Figure 1.2: Autistic Female CBCL Anxiety Predicting ICV-Normalized Left Amygdala Volumes

Note: F = Autistic Female. ASDf = Autistic Female. CBCL = Child Behavioral Checklist.

Overall bivariate regression test: The main effect of CBCL anxiety (B(SE) = 0.35(0.02*), p* = 0.95) was not significant in predicting normalized left amygdala volume.

DISSCUSSION

The goal of the present study was to explore psychopathological and neurodevelopmental differences of ASD and to explore how sex impacts anxiety problems and neurological differences reported in ASD. Our hypothesis that ASDf would display greater anxiety than TDf was supported. However, contrary to predictions, ASDf and ASDm did not differ in CBCL anxiety problems or social behavioral problems (SRS-2). Additionally, our prediction that ASD would display greater left and right amygdala volume (ICV – normalized) was not supported after analyses were conducted. Moreover, our hypotheses that ASDf CBCL anxiety would predict both left and right amygdala volumes (ICV – normalized) did not survive statistical significance.

Previous work assessing the relationship between clinical anxiety and left and right amygdala volumes within ASD have led to mixed findings. Despite the available literature that has examined anxiety and amygdala volumes in ASD, these studies frequently used different methodological approaches in capturing anxiety. Some studies used dimensional approaches (e.g., parent report questionnaire), while others performed categorical assessments in measuring anxiety. As well, new work has argued that "DSM" oriented anxiety measures do not support the identification of anxieties that are unique to ASD populations (e.g., idiosyncratic fears, social confusion of social semantics etc.). In addition, prior work has identified the amygdala to follow a neurodevelopmental trajectory different than TD counterparts. Therefore, future work should aim to 1) develop and utilize instruments that capture autism distinct anxiety in ASD populations to pull apart "DSM" anxiety from autism distinct anxiety; and 2) follow ASD study participants with anxiety longitudinally to be able to document how anxiety impacts ASD participants'

neurodevelopmental growth in general, as well as brain systems crucial for processing social and emotional stimuli.

Limitations

Our study faced several limitations that may have constrained our ability to fully explore the pertinent research objectives at-hand. For objective 1, CBCL anxiety problems are gender normed, and this perhaps struck bias when comparing differences based on sex assigned at birth in ASD. As well, the current data consortium comprises of autistic and non-autistic youth and adolescents aged 8-17. Differences in neurodevelopment and cognitive development, and the variability between age, may have rendered brain and phenotyping data difficult to interpret. In addition, comparing clinical and non-clinical populations on clinical measures such as DSM-oriented anxiety, rendered modeling a difficult challenge to find best fit comparisons. In addition, the ASD population had a mean IQ of above > 100. Although this sample matched with TD counterparts, it does not fully represent the breadth of autistic persons' differences in cognitive ability. ASD individuals with marked intellectual disability (IQ<70) should be considered for future research.

Future work should aim toward de-gendering psychopathological measures such as CBCL anxiety problems and look to carefully consider the meaningfulness of anxiety prevalence in ASD youth.

CONCLUSIONS

The present study provided insight into the psychopathological and neurodevelopmental differences seen in ASD vs TD based on sex assigned at birth. However, we were unable to find meaningful differences in left and right amygdala volumes (ICV – normalized) between groups (ASD vs TD); yet, ASD consistently reported significantly greater anxiety problems relative to controls. We believe these findings reflect limitations in our scope of brain regions of interests and constraints in our data, rather than equal differences between groups.

Of considerable note, we did identify several differences in amygdala volumes between ASD and TD youth in previous work with regards to psychopathology. It is our hope that future work will aim to explore the impacts of sex on psychopathology and neurodevelopment, to illuminate sex differences observed in ASD. Critically, there exists a window where inordinate amount of brain growth is observed in ASD versus non-ASD populations. This finding should warrant caution when comparing ASD populations with age, sex, and IQ matched non-autistic controls. Likewise, researchers exploring the intersection of anxiety and amygdala volumes should use measures that capture not only DSM oriented anxiety disorders, but also autism distinct anxieties that is not otherwise specified in the DSM-V manual. Lastly, existing work exploring brain-based relationships of co-occurring psychiatric conditions in autistic youth has severely lacked participation from ASD individuals assigned female at birth. Recruiting populations in a sex balanced sample is crucial to our ability to understand neurodevelopmental and psychopathological differences for ASD populations, especially youth.

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