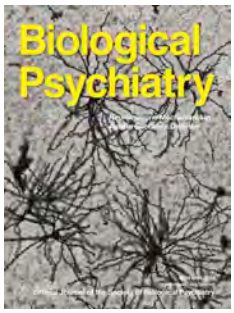

Association of Amygdala Development with Different Forms of Anxiety in Autism Spectrum Disorder

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Abstract

Background: The amygdala is widely implicated in both anxiety and autism spectrum disorder. However, no studies have investigated the relationship between co-occurring anxiety and longitudinal amygdala development in autism. Here, the authors characterize amygdala development across childhood in autistic children with and without traditional DSM forms of anxiety and anxieties distinctly related to autism.

Methods: Longitudinal MRI scans were acquired at up to four timepoints for 71 autistic and 55 typically developing (TD) children (~2.5-12 years, 411 timepoints). Traditional DSM anxiety and anxieties distinctly related to autism were assessed at study Time 4 (~8-12 years) using a diagnostic interview tailored to autism: The Anxiety Disorders Interview Schedule-IV with the Autism Spectrum Addendum. Mixed effects models were used to test group differences at study Time 1 (3.18 years), Time 4 (11.36 years), and developmental differences (age-by-group interactions) in right and left amygdala volume between autistic children with and without DSM or autism distinct anxieties, and TD.

Results: Autistic children with DSM anxiety had significantly larger right amygdala volumes compared to TD at both study Time 1 (5.10% increase) and Time 4 (6.11% increase). Autistic children with autism distinct anxieties had significantly slower right amygdala growth compared to TD, autism-no anxiety, and autism-DSM anxiety groups and smaller right amygdala volumes at Time 4 compared to the autism-no anxiety (-8.13% decrease) and autism-DSM anxiety (-12.05% decrease) groups.

Conclusions: Disparate amygdala volumes and developmental trajectories between DSM and autism distinct forms of anxiety suggest different biological underpinnings for these common, co-occurring conditions in autism.

Introduction

Symptoms of autism spectrum disorder (ASD or autism) include impaired social interaction and communication and restricted repetitive behaviors (1). It is estimated that 42-69% of autistic individuals also meet diagnostic criteria for a clinical anxiety disorder (2,3). Though the amygdala has been widely implicated in both anxiety and autism (4), only three studies have investigated associations between amygdala structure and anxiety within autism (5–7). No studies of autism have investigated the development of the amygdala longitudinally in relation to anxiety, nor the associations between different forms of anxiety and the amygdala in autism.

Clinical anxiety can manifest in several forms, including generalized anxiety disorder (GAD), separation anxiety, specific phobia, and social phobia (henceforth 'DSM anxiety') (1). However, distinguishing anxiety from ASD symptoms is challenging (2,8). Recently developed tools recognize classically defined symptoms of anxiety (e.g., anticipatory anxiety, fearful avoidance) that manifest within contexts that are somewhat unique to autism. These symptoms would not be captured by traditional assessments (9). Such autism-distinct anxieties (henceforth 'distinct anxiety') include fears related to social confusion (as opposed to fear of negative evaluation which is required for a DSM diagnosis of social phobia), uncommon phobias (e.g., specific sounds, facial features), excessive worry related to losing access to materials related to circumscribed interests, and fears of change (3).

Research implicates disruption of the amygdala and its network of connections with the emergence of anxiety (4,10). However, studies of amygdala volume in children and adolescents have proved inconsistent, reporting both larger (11–15) and smaller (16–20) volumes being

associated with anxiety. Others found no associations (21,22) or associations dependent on sex (23,24) or anxiety type (25). These studies utilized a range of anxiety assessments including dimensional measures that are non-specific to the domains of anxiety described in the DSM (e.g., the Child Behavior Checklist [CBCL] and Screen for Child Anxiety Related Emotional Disorders [SCARED]) (26,27), and grouping individuals across DSM anxiety domains according to diagnostic interviews (e.g., Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version [K-SADS-PL]) (28).

Altered amygdala structure and function has also been proposed to underlie social deficits observed in ASD (29–33). MRI studies of autistic children and adolescents have reported larger (31,33–38), smaller (39,40), and no differences (41–43) in amygdala volumes. In developmental terms, converging lines of evidence using MRI and histological (44) methodologies suggest that the amygdala in autism exhibits an initial volumetric overgrowth during early childhood that is then followed by a slowed trajectory of growth into adulthood.

Despite high rates of anxiety in ASD, only three studies have investigated associations of amygdala structure with anxiety in autism. The first found enlarged right amygdala volumes to be associated with increased scores on the CBCL anxious/depressed subscale in autistic children (~4-15 years) (6). A second recent study found no association between the amygdala and CBCL measures in the ABIDE dataset (7). The third compared two groups of autistic children (~7.5-17.5 years) with and without a DSM anxiety diagnosis (5,45), and found autistic children with clinical DSM anxiety had smaller right amygdala volumes compared to those without (5). Thus, two studies indicate a relationship between anxiety and right amygdala volume but taken together

provide contradictory evidence as to whether amygdala volume is larger, smaller, or unrelated to anxiety in autism. Determining the relationship of amygdala volume within autistic development, with and without co-occurring anxiety, may identify biological correlates specific to these conditions and thus provide a valuable prodromal biological marker of anxiety in autism.

In the current study, we characterize and test for anxiety associated differences in the trajectories of volumetric development of the amygdala across childhood (~2.5-12 years), as well as amygdala volumes during early (~3 years) and late (~11 years) childhood in autistic children with and without anxiety disorders. We utilized clinical interviews for both DSM and distinct anxiety to test if these forms of anxiety had different associations with amygdala volume and development in autism. We hypothesize that DSM anxiety will be associated with larger right amygdala volumes and faster development. We further expect anxieties distinctly related to autism will be associated with effects on the amygdala, but since this has not been previously investigated have no a priori prediction as to the nature of this relationship.

Methods and Materials

Participants

Participants were enrolled in the UC Davis MIND Institute Autism Phenome Project, a longitudinal study consisting of MRI scanning at four timepoints, enrollment/baseline at 24-42 months of age (Time 1), follow-up at annual intervals for two time points (Time 2 and 3), and ~9-12 years of age (Time 4). The current study included data from all participants who completed MRI and anxiety assessments at Time 4 (Table 1, Supplementary Figure 1). At Time 1, ASD diagnosis was confirmed using the Autism Diagnostic Observation Schedule-Generic (ADOS-G)

(46) or ADOS-2 (47), the Autism Diagnostic Interview-Revised (ADI-R) (48) and DSM-IV-TR criteria (49). At Time 1, developmental quotient (DQ) was assessed using the Mullen Scales of Early Learning (MSEL) (50). IQ was assessed at Time 4 using the Differential Ability Scales second edition (DAS-II) (51). Informed consent was obtained from the parent or guardian of each participant. All aspects of the study protocol were approved by the University of California Davis Institutional Review Board. See supplementary materials.

Anxiety Assessment

Anxiety was assessed at Time 4 using the Anxiety Disorders Interview Schedule-IV-Parent Interview (ADIS) (45) with the Autism Spectrum Addendum (ADIS-ASA), a semi-structured diagnostic interview designed to differentiate anxiety and autism symptoms (9). The ADIS includes modules relating to separation, social, specific phobia, and generalized anxiety disorders (DSM anxiety). Additionally, the ADIS-ASA assesses forms of anxiety distinctly related to autism, including idiosyncratic fears, fear relating to social confusion, special interest fears, and fears of change (distinct anxiety). For each module a clinical severity rating (CSR) ranging from 0 (no interference) to 8 (severe interference) is prescribed, with 4 being the cutoff for clinical determination of significant interference. Rates of anxiety and assessment within this cohort have been described in detail previously (3). Additional measures were acquired at both Time 1 and Time 4 including; the CBCL (27), Repetitive Behavior Scale (RBS) (52), Short Sensory Profile (SSP) (53), SSP 2 (54), and Social Responsiveness Scale (SRS) (55). See supplementary materials.

Magnetic Resonance Imaging Acquisition and Region of Interest Approach

All T1-weighted structural MRI scans were acquired at the UC Davis Imaging Research Center on a 3T Siemens Trio. Time 1-3 scans were acquired during natural nocturnal sleep (56). Time 4 scans were acquired while participants were awake using previously described methods (57). Distortion corrected, anonymized, and defaced images were uploaded to MRICloud (https://mricloud.org) (58) and segmented into 289 anatomically defined regions using a multi-atlas approach (59). Volumes from the left and right amygdala, and hemispheres, were exported for statistical modeling. See supplementary materials.

Statistical Modeling

Linear mixed effects modeling was performed using *R* v.3.6 (R Core Team, 2019). We first compared differences in amygdala volumes and development associated with anxiety between autistic children with (ADIS DSM CSR>=4) and without DSM anxiety, and with (ADIS-ASA distinct anxiety CSR>=4) and without distinct anxiety. Models included all autistic individuals with categorical factors for DSM and distinct anxiety, and sex, age in months and hemispheric volume as covariates, and individual as a random effect with age as a random slope. Here, overlapping anxieties are accounted for by separate DSM and distinct categorical variables.

A secondary analysis was conducted to compare amygdala volumes and development between five groups of interest: ASD with 1) only DSM anxiety, 2) only distinct anxiety, 3) both DSM and distinct anxiety, 4) no clinical anxiety, and 5) TD without anxiety. Effects of interest for all analyses were mean group differences at the average age at study Time 1 (38.11 months/3.18 years), Time 4 (136.30 months/11.36 years), and differences in developmental trajectories between groups (age-by-group interactions). Age was modeled by selecting from a range of polynomial

terms (-3,-2,-1,-0.5,0.5,1,2,3) that returned the lowest log likelihood (left=0.5, right=-0.5) (60). Analyses were repeated separately for the left and right amygdala. Results for each analysis were corrected for using false discovery rate (FDR) (61) within each hemisphere.

Results

Demographics and Anxiety

Autistic and TD children did not significantly differ ($p>0.05$, two-tailed *t*-test) for age of scan at Time 4 and age of ADIS-ASA assessment. Compared to the TD sample, autistic children had significantly lower overall IQ scores ($p<0.001$) and less longitudinal timepoints per individual ($p=0.008$). Compared to TD, the ASD sample also contained a significantly higher proportion of males ($\chi^2=4.39$, $p=0.03$).

Within the autism sample, 61% (43/71) of participants met diagnostic criteria for at least one form of clinical anxiety, with 45% (32/71) meeting criteria for one or more DSM anxieties, 39% (28/71) meeting criteria for one or more distinct anxieties, and 24% (16/71) meeting criteria for both a DSM and distinct anxiety. In total, 39% (28/71) of autistic children did not reach clinical thresholds for any anxiety type (Table 1). No significant differences (ANOVA $p>0.05$) were found between ASD groups with 1) only DSM anxiety, 2) only distinct anxiety, 3) both DSM and distinct anxiety, and 4) no clinical anxiety in terms of Time 4 scan age, number of MRI timepoints, IQ, or ADOS Calibrated Severity Score (CSS). No significant differences were found for the male-to-female ratio between the four ASD groups ($\chi^2=5.10$, $p=0.16$).

Differences in amygdala volumes associated with clinical DSM anxieties and autism-distinct anxieties

Compared to autistic children without DSM anxieties, those with DSM anxieties (CSR>=4) had larger right amygdala volumes at Time 4 (4.94% mean increase, p=0.017, FDR p=0.038), no statistically significant differences were observed in the left amygdala. Compared to autistic children without distinct anxiety, those with distinct anxiety (CSR>=4) showed smaller right and left amygdala volumes at both Time 1 (Right, -4.02% mean decrease, p=0.019, FDR p=0.38 ; Left, -5.54%, p=0.006, FDR p=0.018), and Time 4 (Right, -4.91% mean decrease, p=0.001, FDR p=0.038 ; Left, -7.14%, p=0.002, FDR p=0.012). Autistic children with autism distinct anxiety also showed a statistical trend (p=0.049, FDR p=0.074) of slower right amygdala development compared to autistic children without distinct anxiety (Figure 1, Table 2).

Differences between autistic children with: only DSM anxiety, only distinct anxiety, both DSM and distinct anxieties, no clinical anxiety, and TD

The above analyses indicate differential associations of amygdala volume between autistic children with DSM (increased volume) and distinct (decreased volume) anxieties. Given the overlap within participants of DSM and distinct anxiety diagnoses, and to investigate the relationship of amygdala volume with TD, we conducted a secondary analysis of amygdala volume and development between five groups of interest; autism with 1) only DSM anxieties (n=16) (ASD-DSM), 2) only distinct anxieties (ASD-distinct) (n=11), 3) both DSM and distinct anxieties (ASD-both anxieties) (n=16), 4) no clinical anxiety (ASD-no anxiety) (n=28), and 5) TD without clinical anxiety (n=55) (Figure 2, Table 3, Supplementary Table 3).

The ASD-DSM group had significantly larger right amygdala volumes at both study Time 1 (5.10% mean increase, p=0.008 FDR, p=0.038) and Time 4 (6.11% mean increase, p=0.009, FDR, p=0.038) compared to TD. No differences in amygdala developmental trajectories between the ASD-DSM group and ASD-no anxiety or TD groups were observed. The ASD-distinct group was found to have a significantly altered developmental trajectory of the right amygdala marked by slower growth compared to the TD (p=0.009, FDR p=0.038), ASD-no anxiety (p=0.009, FDR p=0.038), and ASD-DSM (p=0.006, FDR p=0.038) groups. Slower right amygdala development in the ASD-distinct group resulted in significantly smaller right amygdala volume at Time 4 (11.36 years) compared to the ASD-no anxiety (-8.13% mean decrease, p=0.004, FDR p=0.038) and ASD-DSM (-12.05% mean decrease, p<0.001, FDR p=0.010) groups. No results for the left amygdala reached statistical significance after FDR correction.

Effects of IQ and autism severity on anxiety group differences

No significant differences in IQ or ADOS CSS measures were found between the four autism groups (ANOVA p>0.05) and inclusion of these variables within models did not change statistically significant differences between the groups, nor were they significantly associated with amygdala volumes. Compared to TD, autistic children showed non-significant increases in left (t=1.85, p=0.066) and right (t=1.76, p=0.079) hemispheric volumes. Utilizing the same five group mixed effects model structure, no significant differences between groups in either left or right hemisphere volumes were observed at Time 1, Time 4, or developmentally (i.e., age-by-group interactions), suggesting that our findings are not due to global hemispheric effects (Supplementary Table 1). No significant age-by-group-by-baseline amygdala volume effects between the distinct anxiety and other groups were found (Supplementary Table 2).

Post-hoc Behavioral Analyses

To determine if differences in behaviors associated with autism could explain amygdala differences between DSM and distinct anxiety groups, we performed exploratory post-hoc analyses to investigate differences between the ASD-DSM, ASD-distinct, ASD-both anxieties, and ASD-no anxiety groups in measures including; the CBCL subscales (27), RBS (52), SSP (Time 1) and SSP 2 (Time 4) (53,54), and SRS (55). At Time 4, the ASD-DSM and both anxieties groups had significantly higher CBCL anxious depressed and DSM anxiety problems *t*-scores compared to the ASD-distinct and ASD-no anxiety groups (ANOVA $p<0.05$, Tukey-honest $p<0.05$). These results indicate concordance between CBCL and ADIS assessments of DSM anxiety. The ASD-DSM group also showed significantly higher CBCL internalizing behavior scores compared to the ASD-distinct and ASD-no anxiety groups (ANOVA $p<0.05$, Tukey-honest $p<0.05$). Also, at Time 4 autism groups with anxiety (of any kind) had higher measures of CBCL thought problems, total SSP 2, and total RBS scores compared to the ASD-no anxiety group; however Tukey-honest tests showed these elevated scores to only be significant for the ASD-both anxieties group (CBCL thought problems and RBS) or the ASD-both and ASD-DSM groups (SSP 2) compared to the ASD-no anxiety group (ANOVA $p<0.05$, Tukey-honest $p<0.01$). These findings indicate that autistic children with DSM and/or distinct anxiety experience elevated levels of sensory sensitivities, unusual thought processes and repetitive and restricted behaviors – all potential indications of elevated distress in anxious autistic children (62). The ASD-both group also had significantly higher Time 4 SRS scores compared to the ASD-no anxiety group (ANOVA $p=0.02$, Tukey-honest $p<0.01$). No significant differences between groups in CBCL subscales, total SRS, RBS, or SSP were observed at Time 1 (Supplementary Tables 3-6).

Discussion

The primary aim of this study was to characterize amygdala volume and development across childhood in relation to different types of anxiety in autistic children. We examine two categories of problematic anxiety in ASD: traditional DSM anxieties and anxieties distinct to autism contexts (2,3). Initial analyses comparing autistic children with and without these anxieties revealed DSM anxiety to be associated with enlarged amygdala volumes and distinct anxieties to be associated with smaller amygdala volumes. A second analysis comparing TD children and four subgroups of autistic children (only DSM anxiety, only distinct anxiety, those with both anxieties, and no anxiety) found DSM anxiety to be associated with larger right amygdala volumes compared to TD at both ~3 (Time 1) and ~11 (Time 4) years-of-age but no differences in developmental trajectory. Autistic children with distinct anxiety had slower development of the right amygdala from the ages of ~3-11 compared to TD and other autistic children, and smaller right amygdala volumes at ~11 years of age compared to other autistic children. These results support an association, albeit a complex one, between amygdala volume, ASD, and co-occurring anxiety. Our results also identify a novel association between the development of amygdala volume and anxieties distinctly related to autism.

The only three previous studies of amygdala volumes and anxiety in ASD report conflicting results: finding both larger (6) and smaller (5) right amygdala to be related to anxiety, or no associations (7). The current results support traditional DSM anxieties being associated with larger amygdala volumes. Autism distinct anxieties are estimated to occur at relatively high frequencies in autistic children (3) and have not been previously accounted for in imaging research.

Accordingly, associations of smaller amygdala volumes in autistic children with these distinct anxieties may partially explain inconsistent findings in ASD.

We found that autistic children with anxiety have elevated sensory sensitivities (SSP), repetitive behaviors (RBS), and 'thought problems' (CBCL) which may be indicative of elevated levels of distress. We also noted that autistic children with DSM anxiety had higher CBCL internalizing behavior scores compared to those with distinct anxiety or no anxieties. However, no differences in CBCL internalizing behaviors were observed between the distinct anxiety and no-anxiety groups. Despite finding no differences in measures of core autism features (e.g., ADOS-CSS) between children with and without distinct anxieties, we hypothesize that a latent variable related to the autism phenotype contributes to both autism distinct anxieties and smaller amygdala volumes. Others have reported smaller amygdala volumes in ASD to be associated with decreased joint attention, eye fixation, and emotional face processing speeds (31,42). Emergence of smaller right amygdala volumes due to slower development is indicative of a brain-behavior relationship between amygdala development and onset of distinct anxieties. Replication and future studies are needed to further examine the phenomenology of distinct anxieties and the ways in which their behavioral as well as neurobiological profiles relate to autism and vary from those of DSM anxiety.

We found that autistic children with DSM anxiety had the largest amygdala volumes compared to other groups, with significantly larger right amygdala volumes compared to TD at both ~3 and ~11 years of age. It is important to emphasize that amygdala enlargement in autistic children with DSM anxiety was already present at 3 years-of-age, before clinical anxiety is typically diagnosed. This, and the finding that there were no differences in the trajectory of

amygdala development between ~2.5-12 years would suggest that the process responsible for enlarged amygdala related to DSM anxiety occurred either prenatally or during an early postnatal period. This indicates that enlarged amygdala may be a potential prodromal marker of anxiety in autism. Amygdala enlargement also predating elevated CBCL measures of anxiety in the ASD-DSM group, however this may be attributable to low sensitivity of the CBCL within a sample of autistic children who were likely to be developmentally below the cognitive start age of the CBCL (1.5 years) at study enrollment (3).

Autistic children without anxiety also showed statistical trends toward having larger amygdala volumes compared to TD children while the group of autistic children with both DSM and distinct anxieties had marginally smaller amygdala volumes than autistic children without anxiety. Opposing associations of amygdala volume with DSM (larger) and distinct (smaller) anxieties is also supported by autistic children with both forms of anxiety having amygdala volumes between those of the DSM and distinct anxiety groups. Further studies with larger samples will be needed to confirm these trends. While the underlying mechanisms contributing to smaller and larger amygdala volumes are unclear, larger volumes could be a product of atypical amygdala neurogenesis, which has been noted in ASD (44), while both biological mechanisms (e.g. excitotoxicity) (63) and environmental factors (e.g. poorer quality social interaction) (64) could contribute to stunted amygdala development. The process of untangling these various factors may be difficult since we suspect that neurophenotypic differences between autistic individuals with and without anxiety arise from complex interactions between biological and environmental variables that likely differ on an individual basis.

Significant differences in amygdala volume between autistic individuals with and without autism distinct anxiety were found bilaterally. However, effects between TD and different autism anxiety groups (DSM only, distinct only, both DSM and distinct, and no anxiety) were only significant within the right hemisphere. This is consistent with the two previous studies that have reported relationships between anxiety and amygdala volume in autism (5,6). However, positive associations between both left and right amygdala activation and anxiety have been reported in autism (65,66). Meta analyses have reported predominately leftward lateralization of amygdala activation in response to various emotional processing tasks, which has been postulated to result from increased right amygdala habituation (67,68). Indeed emotional processing functional imaging studies suggest the right amygdala to be more engaged in rapid processing of potentially threatening stimuli, and the left in prolonged stimulus evaluation (67,69,70). Thus, while the amygdala is likely to be affected bilaterally in autistic children with anxiety, a failure of the right amygdala to habituate to anxiolytic stimuli may contribute to the current lateralized findings.

Consistent with the Research Domain Criteria (RDoC) framework (30,66), previous findings suggest that effects of DSM forms of anxiety on the amygdala may cut across diagnostic boundaries. For example, functional MRI studies report increased amygdala activation in response to facial processing tasks and resting state amygdala-prefrontal decoupling to be related to anxiety both in ASD (65,66,71,72) and other populations (4,10,73,74). Furthermore, a recent longitudinal study found positive associations of amygdala volume with anxiety levels from 4-18 years of age in TD (13). The current study was limited by a lack of a control group comprised of non-autistic individuals with clinically significant DSM anxiety. However, we would hypothesize such amygdala enlargement in non-autistic anxious individuals based on our findings of larger

amygdala volumes being associated with DSM anxieties in ASD. In this view, divergent findings of smaller amygdala volumes associated with distinct anxieties may indicate that these manifestations of anxiety are not only distinctly related to autism but are also distinct from traditional DSM classification of anxieties. However, given that distinct anxieties are intrinsically linked to autism and less commonly reach clinical levels in non-autistic individuals, direct comparison of distinct anxieties between autistic and non-autistic groups will be challenging.

This study benefited from a longitudinal design that allowed for the characterization of amygdala growth across early to mid-childhood as well as clinician-based assessments of both traditional DSM and autism distinct anxieties. However, it is important to note limitations. The individual anxiety groups had relatively small sample sizes. Both DSM and distinct anxieties incorporate multiple anxiety subtypes which may, in themselves, have different developmental effects on amygdala volume (25); our sample sizes precluded investigating differences between individual forms of anxiety. Complicating this further is multiple co-occurring anxiety diagnoses. For example, of the 32 autistic children in our sample with a DSM anxiety diagnosis, 37.5% (n=12) meet ADIS criteria for two or more DSM anxiety diagnoses. We assessed autism distinct anxiety using the ADIS-ASA, a diagnostic clinical interview. However, future studies could utilize autism specific parental and patient anxiety reports which may provide more dimensional assessments of anxiety and be easier to widely implement across large samples (75,76). We focused solely on the amygdala due to the structure's broad implications in both anxiety and ASD. However, the amygdala is highly interconnected with other brain regions and thus investigating a broader neural network critical for social and emotional processing in a multimodal fashion would undoubtedly be informative. Higher resolution imaging would afford the ability to investigate individual

contributions of the 13 subnuclei which are exceedingly challenging to segment given the standard ~1mm^3 resolution of current structural MRI scans. Finally, consistent with previous findings in TD (77) and autism (37,78), we found a significant effect of sex, with males having larger amygdala volumes than females. The current study included an insufficient number of autistic females to investigate sex-by-anxiety interactions in amygdala volumes, which is critically important given findings by our group indicating sex specific relationships of amygdala volumes with psychopathology (79), and in the amygdala resting-state connectome (80) in larger samples at younger ages.

In conclusion, the current study aimed to investigate the effect of different forms of anxiety on amygdala volume and development in autistic children. Traditional DSM forms of anxiety were found to be associated with larger right amygdala volumes while anxieties distinctly related to autism were associated with smaller right amygdala volumes and slower right amygdala development. While additional studies are needed to clarify amygdala-anxiety relationships, considering previous findings these results support DSM anxiety having common effects on amygdala volume across diagnostic classifications. Opposing amygdala volumetric relationships between autism distinct anxieties compared to DSM anxieties suggest that these autism related anxiety presentations may be associated with a distinct syndrome of anxiety closely related to the autistic phenotype. Collectively these results indicate that the amygdala is an important brain region for future efforts to identify and stratify those autistic individuals who endure debilitating co-occurring anxiety.

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Table 1: Time Four Participant Demographics				
	ASD (n=71)		TD (n=55)	
Male / Female	57 / 14		34 / 21	
Age	11.18 (1.39)		11.34 (0.67)	
IQ	80.54 (28.77)		111.58 (12.82)	
ADOS CSS	7.5 (1.96)		-	
ADOS social CSS	7.3 (1.73)		-	
ADOS repetitive restricted behavior CSS	7.8 (1.92)		-	
ADIS-DSM Anxiety	32	45%	-	-
General Anxiety Disorder	12	17%	-	-
Separation Anxiety	7	10%	-	-
Social Phobia	5	7%	-	-
Specific Phobia	27	38%	-	-
ADIS-DIST Anxiety	28	39%	-	-
Other Social Fear	5	7%	-	-
Atypical Phobia	10	14%	-	-
Special Interest Fear	4	6%	-	-
Fear of Change	15	21%	-	-
Only ADIS-DSM Anxiety	15	21%	-	-
Only ADIS-DIST Anxiety	11	15%	-	-
Both DSM and DIST Anxiety	17	24%	-	-
Neither DSM or DIST Anxiety	28	39%	55	100%
Scans per participant	3.08 (0.90)		3.49 (0.74)	
Scans at Time 1	67		53	
Scans at Time 2	47		45	
Scans at Time 3	34		39	
Scans at Time 4	71		55	
Total number of Scans	219		192	
Number of participants with four scans	32		35	
Number of participants with three scans	13		12	
Number of participants with two scans	26		8	

Note: IQ and autism diagnostic observation schedule (ADOS) calibrated severity scores (CCS) are given for study time four. ASD=autism spectrum disorder, TD= typically developing, ADIS-DSM = Anxiety Disorders Interview Schedule DSM anxieties, ADIS-DIST = autism spectrum addendum autism distinct anxieties.

Table 2: Amygdala associations with autism distinct and DSM anxiety in autism							
Left Amygdala	**Coef.**	**SE**	**DF**	***t***	***p***	**FDR *p***	**% Diff.**
intercept	1649.86	22.99	144.00	71.77	<0.001	-	-
scan age^0.5	35.03	5.22	144.00	6.71	<0.001	-	-
sex (male reference)	-47.14	40.28	67.00	-1.17	0.246	-	-
hemisphere	0.00	0.00	144.00	4.64	<0.001	-	-
**T1: ASD-noDSM v ASD-DSM	-92.05	32.31	67.00	-2.85	0.006	0.018	5.54 (-0.03 to 10.82)
T1: ASD-noDIST v ASD-DIST	26.69	31.18	67.00	0.86	0.395	0.474	-1.66 (3.86 to -7.48)
**T4: ASD-noDSM v ASD-DSM	-132.67	40.60	67.00	-3.27	0.002	0.012	7.14 (0.91 to 13.02)
T4: ASD-noDIST v ASD-DIST	36.18	39.54	67.00	0.91	0.363	0.474	-2.03 (4.25 to -8.70)
Age-by-Group: ASD-noDIST v ASD-DIST	-7.38	6.02	144.00	-1.23	0.222	0.444	-
Age-by-Group: ASD-noDSM v ASD-DSM	1.72	5.90	144.00	0.29	0.771	0.771	-
Right Amygdala	**Coef.**	**SE**	**DF**	***t***	***p***	**FDR *p***	**% Diff.**
intercept	1811.58	22.13	144.00	81.88	<0.001	-	-
scan age^-0.5	-2171.38	379.38	144.00	-5.72	<0.001	-	-
sex (male reference)	-19.84	39.23	67.00	-0.51	0.615	-	-
hemisphere	0.00	0.00	144.00	5.89	<0.001	-	-
**T1: ASD-noDSM v ASD-DSM	-74.47	31.08	67.00	-2.40	0.019	0.038	4.02 (-0.92 to 8.73)
T1: ASD-noDIST v ASD-DIST	37.26	29.96	67.00	1.24	0.218	0.218	-2.07 (2.80 to -7.19)
**T4: ASD-noDSM v ASD-DSM	-140.37	40.03	67.00	-3.51	0.001	0.006	6.91 (1.33 to 12.22)
**T4: ASD-noDIST v ASD-DIST	95.25	39.02	67.00	2.44	0.017	0.038	-4.94 (0.86 to -11.09)
*Age-by-Group: ASD-noDIST v ASD-DIST	863.31	434.14	144.00	1.99	0.049	0.074	-
Age-by-Group: ASD-noDSM v ASD-DSM	-759.75	425.12	144.00	-1.79	0.076	0.091	-

Note: Regression table for models of left and right amygdala volume between children autism spectrum disorder (ASD) with and without DSM anxiety (ASD-DSM, ASD-noDSM) and with and without autism distinct anxiety anxiety (ASD-DIST, ASD-noDIST). Effects of interest are shown for mean age of study Time 1 (T1, 38.11 months/3.18 years) and Time 4 (T4, 136.30 months/11.36 years) as well as developmental differences (age-by-group interactions). Note that here some individuals within the DSM/DIST ANX groups have duel DSM and distinct anxiety diagnoses, also individuals within the no DSM group may have distinct CSR scores >=4 (and vice versa for the no DIST group). These overlapping anxieties are accounted for by modeling separate DSM and distinct categorical variables. Reference group is always indicated first. Coef=coefficient, SE=standard error, DF=degrees of freedom, *p*=uncorrected *p* value, FDR *p*= false discovery rate adjusted *p* value. % Diff. = percent difference in amygdala volume between groups at Time 1 and Time 4 including (95% confidence range of percent differences). *effects of interest $p<0.05$, **effects of interest FDR $p<0.05$

Table 3: Amygdala associations between ASD groups with different anxiety types and TD							
Left Amygdala	**Coef.**	**SE**	**DF**	***t***	***p***	**FDR *p***	**% Diff.**
(Intercept)	1625.44	19.27	277	84.33	<0.001	-	-
scan age^0.5	26.95	3.63	277	7.42	<0.001	-	-
sex (male reference)	-83.57	26.05	122	-3.21	0.002	-	-
hemisphere	0.00	0.00	277	7.29	<0.001	-	-
T1:TD v ASD-no ANX	35.58	29.37	122	1.21	0.228	0.474	-2.22 (2.85 to -7.52)
T1:TD v ASD-DIST ANX	-63.53	41.79	122	-1.52	0.131	0.393	3.97 (-2.94 to 10.59)
T1:TD v ASD-DSM ANX	40.29	34.77	277	1.16	0.248	0.474	-2.52 (3.31 to -8.60)
T1: TD v ASD-DSM & DIST ANX	-12.22	33.21	277	-0.37	0.713	0.832	0.76 (-5.07 to 6.35)
*T1: ASD-no ANX v ASD-DIST ANX	-99.11	44.87	122	-2.21	0.029	0.164	6.05 (-1.52 to 13.20)
T1: ASD-no ANX v ASD-DSM ANX	4.71	38.10	277	0.12	0.902	0.933	-0.29 (6.13 to -7.10)
T1: ASD-no ANX v ASD-DSM & DIST ANX	-47.80	37.49	277	-1.28	0.203	0.469	2.92 (-3.62 to 9.09)
*T1: ASD-DIST ANX v ASD-DSM ANX	103.82	48.40	277	2.15	0.033	0.164	-6.75 (1.96 to -16.36)
T1: ASD-DIST ANX v ASD-DSM & DIST ANX	51.31	47.85	277	1.07	0.285	0.474	-3.34 (5.05 to -12.58)
T1: ASD-DSM ANX v ASD-DSM & DIST ANX	-52.51	39.31	278	-1.34	0.183	0.457	3.20 (-4.11 to 9.99)
T4:TD v ASD-no ANX	66.48	36.17	122	1.84	0.069	0.294	-3.80 (2.00 to -9.89)
T4:TD v ASD-DIST ANX	-55.01	51.06	122	-1.08	0.283	0.474	3.14 (-4.70 to 10.61)
*T4:TD v ASD-DSM ANX	96.46	44.39	277	2.17	0.031	0.164	-5.51 (1.34 to -12.71)
T4: TD v ASD-DSM & DIST ANX	-15.00	41.96	277	-0.36	0.721	0.832	0.86 (-5.91 to 7.30)
*T4: ASD-no ANX v ASD-DIST ANX	-121.49	54.75	122	-2.22	0.028	0.164	6.69 (-1.71 to 14.56)
T4: ASD-no ANX v ASD-DSM ANX	29.98	48.39	277	0.62	0.536	0.699	-1.65 (5.69 to -9.49)
T4: ASD-no ANX v ASD-DSM & DIST ANX	-81.48	46.76	277	-1.74	0.083	0.310	4.49 (-2.89 to 11.40)
*T4: ASD-DIST ANX v ASD-DSM ANX	151.47	60.34	277	2.51	0.013	0.164	-8.94 (1.02 to -17.97)
T4: ASD-DIST ANX v ASD-DSM & DIST ANX	40.01	59.05	277	0.68	0.499	0.699	-2.36 (7.01 to -12.81)
*T4: ASD-DSM ANX v ASD-DSM & DIST ANX	-111.46	51.43	278	-2.17	0.031	0.164	6.04 (-2.21 to 13.62)
Age-by-group: TD v ASD-no ANX	5.61	5.22	277	1.08	0.283	0.474	-
Age-by-group: TD v ASD-DIST ANX	1.55	7.45	277	0.21	0.835	0.895	-
Age-by-group: TD v ASD-DSM ANX	10.21	6.51	277	1.57	0.118	0.393	-
Age-by-group: TD v ASD-DSM & DIST ANX	-0.51	6.30	277	-0.08	0.936	0.936	-
Age-by-group: ASD-no ANX v ASD-DIST ANX	-4.07	8.05	277	-0.51	0.614	0.767	-
Age-by-group: ASD-no ANX v ASD-DSM ANX	4.59	7.18	277	0.64	0.523	0.699	-
Age-by-group: ASD-no ANX v ASD-DSM & DIST ANX	-6.12	6.99	277	-0.88	0.382	0.573	-
Age-by-group: ASD-DIST ANX v ASD-DSM ANX	8.66	8.94	277	0.97	0.333	0.526	-
Age-by-group: ASD-DIST ANX v ASD-DSM & DIST ANX	-2.05	8.79	277	-0.23	0.815	0.895	-
Age-by-group: ASD-DSM ANX v ASD-DSM & DIST ANX	-10.72	8.01	278	-1.34	0.182	0.457	-

Right Amygdala	**Coef.**	**SE**	**DF**	***t***	***p***	**FDR *p***	**% Diff**
(Intercept)	1763.83	18.38	277	95.96	<0.001	-	-
Scan age^-0.5	-2219.73	273.45	277	-8.12	<0.001	-	-
sex (male reference)	-83.95	25.18	122	-3.33	0.001	-	-
hemisphere	0.00	0.00	277	8.10	<0.001	-	-
T1:TD v ASD-no ANX	49.78	27.90	122	1.78	0.077	0.210	-2.86 (1.66 to -7.56)
T1:TD v ASD-DIST ANX	11.06	39.92	122	0.28	0.782	0.835	-0.64 (5.34 to -6.85)
**T1:TD v ASD-DSM ANX	88.78	33.17	277	2.68	0.008	0.038	-5.10 (0.13 to -10.54)
T1: TD v ASD-DSM & DIST ANX	34.09	31.79	277	1.07	0.284	0.474	-1.96 (3.11 to -7.22)
T1: ASD-no ANX v ASD-DIST ANX	-38.71	42.90	122	-0.90	0.369	0.553	2.16 (-4.57 to 8.55)
T1: ASD-no ANX v ASD-DSM ANX	39.00	36.36	277	1.07	0.284	0.474	-2.18 (3.52 to -8.18)
T1: ASD-no ANX v ASD-DSM & DIST ANX	-15.68	35.96	277	-0.44	0.663	0.796	0.88 (-4.93 to 6.40)
T1: ASD-DIST ANX v ASD-DSM ANX	77.72	46.33	277	1.68	0.095	0.236	-4.44 (2.88 to -12.38)
T1: ASD-DIST ANX v ASD-DSM & DIST ANX	23.03	45.94	277	0.50	0.616	0.796	-1.31 (5.77 to -9.01)
T1: ASD-DSM ANX v ASD-DSM & DIST ANX	-54.68	38.51	278	-1.42	0.157	0.313	2.99 (-3.32 to 8.92)
T4:TD v ASD-no ANX	58.92	35.88	122	1.64	0.103	0.238	-3.09 (2.19 to -8.61)
*T4:TD v ASD-DIST ANX	-101.14	50.72	122	-1.99	0.048	0.145	5.30 (-1.78 to 12.06)
**T4:TD v ASD-DSM ANX	116.74	44.11	277	2.65	0.009	0.038	-6.11 (0.15 to -12.66)
T4: TD v ASD-DSM & DIST ANX	42.66	41.50	277	1.03	0.305	0.481	-2.23 (3.68 to -8.42)
**T4: ASD-no ANX v ASD-DIST ANX	-160.07	54.42	122	-2.94	0.004	0.038	8.13 (0.47 to 15.34)
T4: ASD-no ANX v ASD-DSM ANX	57.82	48.13	277	1.20	0.231	0.433	-2.94 (3.87 to -10.17)
T4: ASD-no ANX v ASD-DSM & DIST ANX	-16.26	46.34	277	-0.35	0.726	0.835	0.83 (-6.02 to 7.28)
**T4: ASD-DIST ANX v ASD-DSM ANX	217.88	60.02	277	3.63	<0.001	0.010	-12.05 (-2.58 to -20.36)
**T4: ASD-DIST ANX v ASD-DSM & DIST ANX	143.80	58.60	277	2.45	0.015	0.053	-7.95 (1.05 to -17.92)
T4: ASD-DSM ANX v ASD-DSM & DIST ANX	-74.08	51.22	278	-1.45	0.149	0.313	3.66 (-3.85 to 10.61)
Age-by-group: TD v ASD-no ANX	-119.80	386.05	277	-0.31	0.757	0.835	-
**Age-by-group: TD v ASD-DIST ANX	1469.87	557.89	277	2.63	0.009	0.038	-
Age-by-group: TD v ASD-DSM ANX	-366.28	475.37	277	-0.77	0.442	0.631	-
Age-by-group: TD v ASD-DSM & DIST ANX	-112.22	459.27	277	-0.24	0.807	0.835	-
**Age-by-group: ASD-no ANX v ASD-DIST ANX	1589.67	603.15	277	2.64	0.009	0.038	-
Age-by-group: ASD-no ANX v ASD-DSM ANX	-246.48	527.62	277	-0.47	0.641	0.796	-
Age-by-group: ASD-no ANX v ASD-DSM & DIST ANX	7.58	513.01	277	0.01	0.988	0.988	-
**Age-by-group: ASD-DIST ANX v ASD-DSM ANX	-1836.15	663.74	277	-2.77	0.006	0.038	-
*Age-by-group: ASD-DIST ANX v ASD-DSM & DIST ANX	-1582.09	652.28	277	-2.43	0.016	0.053	-
Age-by-group: ASD-DSM ANX v ASD-DSM & DIST ANX	254.07	583.68	278	0.44	0.664	0.796	-

Note: Regression results for models of left and right amygdala volume between children autism spectrum disorder with only DSM anxiety (ASD-DSM ANX), only autism distinct anxiety (ASD-DIST ANX), both anxieties (ASD-DSM & DIST ANX), without anxiety (ASD-no ANX), and typical development (TD). Effects of interest are shown for mean age of study Time 1 (T1, 38.11 months/3.18 years) and Time 4 (T4, 136.30 months/11.36 years) as well as developmental differences (age-by-group interactions). Reference group is always indicated first. Regressions were

repeated switching reference group to provide all group comparisons. Coef=coefficient, SE=standard error, DF=degrees of freedom, *p*=uncorrected *p* value, FDR *p*=false discovery rate adjusted *p* value, % Diff. = percent difference in amygdala volume between groups at Time 1 and Time 4 including (95% confidence range of percent differences). *effects of interest p<0.05, **effects of interest FDR p<0.05

Figure 1: Associations between amygdala volume and DSM and autism distinct anxieties Longitudinal development of left (A) and right (B) amygdala volumes are plotted for autistic children with and without DSM anxiety (ASD-DSM ANX, ASD-no DSM ANX). Compared to autistic children without DSM anxiety, those with DSM anxiety showed larger right amygdala volumes at both Time 1 (5.87% larger, p=0.005, FDR p=0.048) and Time 4 (6.15% larger, p=0.005, FDR p=0.048). Compared to autistic children without autism distinct anxieties, those with distinct anxieties (ASD-DIST ANX, ASD-no DIST ANX) showed statistical trends (p<0.05, FDR p>0.05) of (C) toward smaller left amygdala volumes at both Time 1 (-5.44% smaller, p=0.024) and Time 4 (-6.75% smaller, p=0.011) and (D) right amygdala volumes at Time 4 (-5.75% mean decrease, p=0.020) compared to ASD-noANX. Vertical dotted lines indicate mean ages at study Time 1 (38.11 months/3.18 years) and 4 (136.30 months/11.36 years). *Note that here some individuals within the DSM/DIST ANX groups have duel DSM and distinct anxiety diagnoses, also individuals within the no DSM group may have distinct CSR scores >=4 (and vice versa for the no DIST group). These overlapping anxieties are accounted for by modeling separate DSM and distinct categorical variables.

Figure 2: Associations between amygdala volume and different forms of anxiety in autism spectrum disorder Longitudinal development of left (A) and right (B) amygdala volumes are plotted for autistic children with only DSM anxiety (ASD-DSM ANX), with only autism distinct anxiety (ASD-DIST ANX), with both DSM and distinct anxieties (ASD-BOTH ANX), without anxiety (ASD-noANX), and typically developing children without anxiety (TD). Vertical dotted lines indicate mean ages at study Time 1 (38.11 months/3.18 years) and Time 4 (136.30 months/11.36 years). ASD-DSM ANX was found to have significantly larger right amygdala

volumes at both study Time 1 (4.59% larger, p=0.012 FDR, p=0.044) and Time 4 (5.19% larger, p=0.011, FDR, p=0.044) compared to TD. ASD-DIST ANX had a significantly altered developmental trajectory of the right amygdala marked by slower growth compared to the TD (p=0.009, FDR p=0.044), ASD-noANX (p=0.009, FDR p=0.044), and ASD-DSM ANX (p=0.007, FDR p=0.044) groups which resulted in significantly smaller right amygdala volumes at Time 4 compared to the ASD-no anxiety (-8.85% smaller, p=0.004, FDR p=0.044), ASD-DSM (-11.04% smaller, p<0.001, FDR p=0.013), and ASD-both anxieties (-8.26% smaller, p=0.011, FDR p=0.044) groups.