Title: Restructuring of amygdala subregion apportion across adolescence

Abbreviated Title: Amygdala subnuclei development across adolescence

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Abstract

Total amygdala volumes continue to increase from childhood to young adulthood. Interestingly, postmortem studies have found postnatal neuron numbers increase in a nuclei specific fashion across development, suggesting amygdala maturation may involve changes to its composition. Thus, the goal of this study was to examine amygdala subregion apportionment in vivo and examine if these patterns were associated with age, sex, body mass index (BMI), and pubertal status in a large sample of typically developing adolescents (N=421, 44% female, ages 10-17 years). We utilized the CIT168 atlas to examine the relative volume fraction (RVF) of 9 subregions within each hemisphere of the amygdala. Generalized Additive Mixed Models (GAMM) were used to assess how demographic variables (e.g. age, sex) and physical development (e.g. BMI and pubertal status) were associated with amygdala RVFs. Results showed that age associations varied significantly by sex for the RVFs of the lateral (LA), basolateral ventral and paralaminar subdivision (BLVPL), central nucleus (CEN), and amygdala transition areas (ATA). While pubertal development was found to be associated with RVFs in the BLVPL, CEN, and ATA in males, best-fit model comparisons revealed that age was the best predictor of relative volumes of these subregions. These results suggest that the relative apportionment of the amygdala further develops with age in males across adolescence. These findings may help elucidate how sex differences could impact the prevalence of mental health disorders that arise during this adolescent period of development.
Significance Statement: Given the heterogeneity of cytoarchitecture, connectivity, and function between amygdala subregions, naturally more research is needed to understand amygdala composition across human adolescence. Our findings show that males, but not females, demonstrate amygdala composition development across the adolescent years of 10 to 17. In males, there is a relative expansion of the lateral and central subregions, but a contraction of the basolateral ventral and paralaminar subdivision and amygdala transition areas within the amygdala. Distinct maturation patterns of the amygdaloid complex across adolescence may be an important mechanism contributing to sex differences in emotional processing as well as the onset, prevalence, and symptomatology for affective disorders that typically emerge during this developmental period.
Introduction

The amygdala is a collection of nuclei located in the temporal lobe, with extensive connections to the cerebral cortex (Amaral and Price, 1984; Barbas and De Olmos, 1990; Ghashghaei and Barbas, 2002). The heterogeneous structure and function of the amygdala nuclei play a vital role in mediating a number of cognitive, affective, and motivational processes (Baxter and Murray, 2002; Hariri et al., 2002; Meyer-Lindenberg et al., 2005; Raznahan et al., 2011; Bzdok et al., 2013; Tottenham andGabard-Durnam, 2017). Cytoarchitecture and lesion studies have helped determine how these diverse groupings of amygdala neurons mediate specific processes (Krettek and Price, 1978; Amaral and Price, 1984; Ghashghaei and Barbas, 2002; Amunts et al., 2005; Solano-Castiella et al., 2011). Previous studies have shown the basal and lateral nuclei process high-level sensory input and emotional regulation (Sananes and Davis, 1992; Wan and Swerdlow, 1997; Schoenbaum et al., 1999), while the central and basolateral nuclei are involved in reward learning and food intake (Killcross et al., 1997; Rollins and King, 2000; Baxter and Murray, 2002; Ambroggi et al., 2008). Moreover, the region closest to the ventral horn, known as the paralaminar nucleus contains neurons that continue to mature and migrate into adulthood (Amaral and Price, 1984; Bernier et al., 2002; Tosevski et al., 2002; deCampo and Fudge, 2012); this region’s potential for regional neural plasticity (deCampo and Fudge, 2012) may be important for modulating amygdala apportionment.

When treating the amygdala as a singular unit, total amygdala volumes continue to increase from childhood to young adulthood, with distinct developmental patterns seen based on sex and pubertal stage (Giedd et al., 1996; Bramen et al., 2011; Herting et al., 2014; Wierenga et al., 2014; Herting et al., 2018; Wierenga et al., 2018). However, a recent postmortem study (N=24 neurotypical brains, ages 2-48 years) found that neuron numbers increase in the amygdala, but do so in a nucleus specific manner (Avino et al., 2018). These findings suggest that neuronal increase in specific nuclei may prompt relative changes in amygdala nuclei apportionment with
development. Accordingly, our study aimed to test the hypothesis that the relative ratio of individual nuclei to the total amygdala volume, or the relative volume fraction (RVF), develops across human adolescence. While previous atlases utilized ex vivo brain tissue to delineate the amygdala into smaller regions of interests (ROIs) (Amunts et al., 2005; Saygin et al., 2017), we implemented a novel high-resolution probabilistic atlas, known as the CIT168, based on in vivo MRI data (Tyszka and Pauli, 2016; Pauli et al., 2018). Using this approach, we segmented the amygdala into 9 distinct bilateral ROIs for 421 adolescents (n=186 females, ages 10-17 years), including the lateral nucleus (LA), basolateral dorsal and intermediate subdivision (BLDI), basolateral ventral and paralaminar subdivision (BLVPL), basomedial nucleus (BM), cortical and medial nuclei (CMN), central nucleus (CEN), anterior amygdala area (AAA), amygdala transition areas (ATA), and amygdalo striatal transition area (ASTA) (Table 1).

Choosing predictors based on previous research (Rollins and King, 2000; Baxter and Murray, 2002; Herting et al., 2014; Wierenga et al., 2014; Janak and Tye, 2015; Tyszka and Pauli, 2016; Herting et al., 2018; Wierenga et al., 2018), we explored how age, sex, body mass index (BMI), and pubertal status were associated with amygdala composition in adolescents. Given that the basolateral nucleus increases innervation with the prefrontal cortex during adolescent neurodevelopment (Cunningham et al., 2002) and the paralaminar’s potential for postnatal neuroplasticity (deCampo and Fudge, 2012), we hypothesized that lateral, basal, and paralaminar subregions would be larger with age across adolescence. We also hypothesized that a higher BMI would correlate with the central and basal subregions, given their involvement in reward learning (Killcross et al., 1997; Rollins and King, 2000; Baxter and Murray, 2002; Ambroggi et al., 2008). Ultimately, understanding how the human amygdala develops throughout adolescence may help discern developmental changes seen in social-emotional and reward-related behavior, as well as identify risk factors for mental health disorders.
Materials and Methods

Participants and Measures

This study incorporated cross-sectional data from 421 adolescents (n=186 females), ages 10 to 17 years, from ongoing research studies at Oregon Health & Science University. A comprehensive telephone interview was conducted to determine eligibility for all participants, and written consent and assent were obtained from each participating adolescent and at least one of their biological parents. All participants were right-handed and free of neurological, neurodevelopmental, and/or psychological diagnoses. Detailed exclusionary criteria can be found elsewhere (Alarcon et al., 2015; Scheuer et al., 2017; Morales et al., 2018).

Based on prior research (Rollins and King, 2000; Baxter and Murray, 2002; Herting et al., 2014; Wierenga et al., 2014; Janak and Tye, 2015; Tyszka and Pauli, 2016; Herting et al., 2018; Wierenga et al., 2018), we considered four primary biological and physical factors for each participant: age, sex, pubertal status, and BMI. Pubertal status was determined by self-report using the Pubertal Development Scale (PDS) (Petersen et al., 1988), with scores for each of the 5 questions ranging from 1 (not started) to 4 (development seems complete). Scores across the items were averaged to a single comprehensive score. Weight and height were also obtained on-site within 1-week of the scan session. BMI was calculated using the Centers for Disease Control and Prevention’s BMI Percentile Calculator for Child and Teen English Version (http://nccd.cdc.gov/dnpabmi/Calculator.aspx) by providing participant birth date, date of measurement, sex, height (to nearest 0.1 cm) and weight (to nearest 0.1 kg). BMI z-scores (BMIz), which correspond to growth chart percentiles, were then calculated to reflect the relative weight of the individual using the appropriate reference standard based on the individual’s age and sex (Must and Anderson, 2006).

MRI Data Collection and Preprocessing
A whole-brain T1-weighted MRI scan was acquired for each participant on the same 3 Tesla MRI system (Magnetom Tim Trio, Siemens Medical Solutions, Erlangen, Germany) using a 12-channel head coil at the Oregon Health & Science University's Advanced Imaging Research Center (TR = 2300 ms, TE = 3.58 ms, TI = 900 ms, flip angle = 10°, 256x240 matrix, voxel size = 1 mm x 1 mm x 1.1 mm). Raw images were quality checked for motion and given a rating of 1 (pass), 2 (review), or 3 (fail) (Backhausen et al., 2016). Using the Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) version 5.0 (Smith et al., 2004; Woolrich et al., 2009; Jenkinson et al., 2012), each brain image was first reoriented to standard orientation using FSL's `fslreorient2std` function. Images were then automatically cropped to reduce lower head and neck using FSL's `robustfov` tool and rigid-body AC-PC aligned. Using the `antsBrainExtraction` function from the Advanced Normalization Tools (ANTs, Version 2.1.0.post691-g9bc18)(Avants et al., 2011), each image was skull-stripped to allow for an N4 Bias Field Correction (Tustison et al., 2010) on the whole-brain image.

Amygdala Segmentation

Details of the in vivo amygdala probabilistic atlas construction, validation, estimates of individual differences, and comparison with previous atlas’ have been previously published (Tyszka and Pauli, 2016; Pauli et al., 2018). Each participant’s image was registered to the CIT168 atlas using a B-spline bivariate symmetric normalization (SyN) diffeomorphic registration algorithm from ANTs (Avants et al., 2007). Implementation of the inverse diffeomorphism resulted in a probabilistic segmentation of each participant’s left and right total amygdala estimates, as well as the following 9 bilateral regions of interest (ROI): lateral nucleus (LA); dorsal and intermediate divisions of the basolateral nucleus (BLDI); ventral division of the basolateral nucleus and paralaminar nucleus (BLVPL); basomedial nucleus (BM); central nucleus (CEN); cortical and medial nuclei (CMN); amygdala transition areas (ATA); amygdalo striatal transition area (ASTA); and anterior amygdala area (AAA). A 2-Dimensional visual representation of the amygdala
subregion segmentation on a representative subject can be seen in Figure 1. To fully
demonstrate the CIT168 segmentation, overlay images of coronal slices through the entire rostral-
caudal extent of the amygdala for four subjects are presented in Figure 2, with boundary outlines
(without an overlay) presented in Figure 2-1. The subjects were randomly chosen to cover the
distributions of our age range, including 1 male and 1 female from both the early and older
adolescent periods. Descriptions of each subregion can be found in Table 1. A relative volume
fraction (i.e. a proportion estimate) was computed for each ROI by normalizing it to the respective
total amygdala volume in each hemisphere (Relative Volume Fraction =
ROI probabilistic volume/total amygdala probabilistic volume). The quality of all amygdala
segmentations was confirmed visually (A.F.M.).

Contrast-to-Noise Ratio (CNR) Calculations for Segmentation Accuracy

In the creation and validation of the CIT168 atlas, Tyszka and Pauli (2016) establish that
a CNR >1 provides a robust volume estimation of the ground truth volumes of an estimate.
Thus, the intensity contrast within each hemisphere of the amygdala was estimated from the
interquartile range of intensities within the entire amygdala from each subject’s T1-weighted
image. The standard deviation (SD) of the noise was estimated from the residual signal
obtained from the subtracted T1-weighted atlas template image from each subject’s T1-
weighted image. The interquartile range (IQR) was then divided by the mean residual noise SD
to generate the CNR for each individual.

Statistical Analysis

Data were analyzed in R (version 3.5.1). Linear regressions (M1) were utilized to examine
the associations between age and intracranial volume (ICV), ICV and BMIz, BMIz and age, BMIz
and PDS, PDS and ICV. These associations were assessed across all participants and between
males and females to see if the associations were significantly different by sex:
To examine if total amygdala and amygdala nuclei volume composition (i.e. RVFs) related to age, sex, BMIz, and pubertal status, we employed a Generalized Additive Mixed Model (GAMM) implemented by the *mgcv* package (version 1.8-24 in R version 3.5.1, R Core Team, 2018). Given that this developmental period shows non-linear subcortical brain volume growth patterns (Wierenga et al., 2014; Herting et al., 2018), a GAMM approach was chosen as it allows for data-driven estimation of non-linear associations (with linearity as a special case), using ‘smooth’ functions, s(), in place of linear terms. To examine the association between age and amygdala nuclei composition, as well as determine if these associations vary by sex, RVF of each amygdala subregion was modeled independently using a GAMM (M2) with fixed effects including smooth terms for age and age-by-sex (s1 and s2, respectively), as well as a linear term for sex, hemisphere, BMIz, ICV, and a random intercept (Ui) for participant i:

\[ M2: RVF_{ij} = \beta_0 + s_1(Age_i) + \beta_1 Male_i + s_2(Age_i) \times Male_i + \beta_2 Hemisphere_{ij} + \beta_3 BMI_i + \beta_4 ICV_i + U_i + \epsilon_{ij} \]

where RVFij is the relative volume fraction (RVF) defined for each subject, i, in either the left or right hemisphere, j. Each smooth term is a shrinkage version of a cubic regression spline with four equally spaced knots.

Given that markers of pubertal development have been shown to relate to total amygdala volumes across adolescence (Goddings et al., 2014; Herting et al., 2014; Wierenga et al., 2018), we then utilized a model building strategy to determine if age, pubertal development, or their combination best predicted amygdala subregion RVFs across adolescence. Given that pubertal development follows a different age-related trajectory in males versus females and physical changes are distinct in males (e.g. facial hair, testes development) and females (e.g. breast development, menstruation) (Berenbaum et al., 2015), these analyses were performed in each
sex separately. First, in each sex we examined the smooth effect of age (M3). Next, we examined the smooth effect of pubertal stage (M4). Lastly, we examined both the smooth effects of age and pubertal stage as well as the interaction term of age-by-pubertal stage (M5), with smooths implemented by tensor product interactions, allowing for main effects and the interaction. Each model also included the fixed effects of BMIz, hemisphere, ICV, and a random intercept (Ui) for participant i.

\[
M_3: \text{RVF}_{ij} = \beta_0 + s_1(\text{Age}_i) + \beta_2\text{Hemisphere}_{ij} + \beta_3\text{BMIz}_i + \beta_4\text{ICV}_i + U_i + \varepsilon_{ij}
\]

\[
M_4: \text{RVF}_{ij} = \beta_0 + s_1(\text{Pubertal Stage}_i) + \beta_2\text{Hemisphere}_{ij} + \beta_3\text{BMIz}_i + \beta_4\text{ICV}_i + U_i + \varepsilon_{ij}
\]

\[
M_5: \text{RVF}_{ij} = \beta_0 + s_1(\text{Age}_i) + s_2(\text{Pubertal Stage}_i) + s_3(\text{Age}_i, \text{Pubertal Stage}_i) + \beta_2\text{Hemisphere}_{ij} + \beta_3\text{BMIz}_i + \beta_4\text{ICV}_i + U_i + \varepsilon_{ij}
\]

Akaike Information Criterion (AIC) and Likelihood ratio tests (p < 0.05) were used to compare model fits. To reduce type I error, each set of models across the 9 ROIs were corrected for multiple comparisons using the Bonferroni correction method (Bonferroni, 1936), with p-values <0.0056 deemed significant.

**Results**

Males and females did not differ in age, BMI, or pubertal status (PDS), though on average, males had a significantly larger ICV compared to females (β=121231, p=<0.0001) (Table 2A). No significant associations were detected between age and ICV, ICV and BMIz, BMIz and age, and PDS and ICV across all participants (Table 2B). A larger BMIz score was associated with a smaller ICV in males (p=0.04), whereas larger BMIz was associated with higher PDS scores in females (p=0.002). The associations between these variables did not significantly differ between the sexes (p’s>0.05) (Table 2B). The mean SD of residual signal obtained from the CIT168 mask and the T1-weighted image of the whole amygdala was 24 for the right and left hemispheres. The mean lower and upper quartile intensities within the amygdala were 276 and 309 (IQR=33) for
the right hemisphere and 275 and 308 (IQR=33) for the left hemisphere, with the residual noise
standard deviations of 0.20 for both hemispheres. Therefore, the average CNR was 1.4 for the
amygdala in both hemispheres in our sample, suggesting the current study has sufficient CNR
necessary to implement reliable estimates utilizing the diffeomorphic approach (Tyszka and Pauli,
2016).

Age and sex differences in amygdala composition

RVOs of each subregion using the CIT168 are summarized by hemisphere and sex in

**Figure 3** and **Table 3**. From largest to smallest, subregion absolute volumes were on average
332-391 mm$^3$ for the lateral nucleus (~20-21% of amygdala volume); 198-to 230 mm$^3$ for the BLDI
(~12-13% of amygdala volume); 171-195 mm$^3$ for the CMN (~11% of the amygdala volume); 118-
141 mm$^3$ for the BLVPL (~7-8% of the amygdala volume); 114 to 131 mm$^3$ for the BM (~7% of the
amygdala volume); 93-111 mm$^3$ for the ATA (~5-6% of the amygdala volume); 69-77 mm$^3$ for the
ASTA (~4 % of the amygdala volume); 63-71 mm$^3$ for the AAA (~3-4% of the amygdala volume);
47-53 mm$^3$ for the CEN (~3% of the amygdala volume).

GAMM model results examining the associations between amygdala subregion RVF and
age, sex, hemisphere, BMIz, ICV, and age-by-sex interactions are presented in **Table 4**. A
significant age-by-sex interaction was detected for the LA (Adj $R^2$=.06), BLVPL (Adj $R^2$=.13), CEN
(Adj $R^2$=.10), and ATA (Adj $R^2$=.12) (**Figure 4**). The LA and CEN show a relative increase of the
total amygdala volume (as indexed by larger RVF values) with age in males, whereas females
show no changes in the relative volume of these amygdala subregions with age. In contrast, the
BLVPL and ATA show a relative decrease in relation to the total amygdala volume (e.g. smaller
RVFs) with age in males, whereas again no relationship is seen in females. The relative volumes
of the BLDI, BM, CMN, and AAA did not relate to age, sex, or their interaction. In addition, no
significant relationships were seen between any of the 9 subregions and BMIz.
Pubertal development and amygdala composition in males and females

GAMM model outputs for age (M3), puberty (M4), and age-by-puberty (M5) for each RVF in each sex separately are presented in Table 5 for males and Table 6 for females. For females, no significant age, puberty, or age-by-puberty associations were seen for any of the 9 amygdala subregions. In males, age was again found to be significantly associated with RVFs of the BLVPL, CEN, and ATA (M3: p's ≤ 0.005), and trending for LA (M3: p's ≤ 0.01). In addition, puberty was found to significantly relate to RVFs of the BLVPL, CEN, and ATA (M4: p's ≤ 0.005). There were no age-by-pubertal interactions that were significant for any of the 9 amygdala subregions after correcting for multiple comparisons; though a trend was seen for the BLVPL (age-by-PDS: p=0.05; Adj R²: 0.14). For the BLVPL and ATA, best-fit model comparisons showed that the age and puberty model was significantly better than the model including only puberty (M4 vs. M5: p's>0.05); however, the age and puberty model was not a significantly better model than age alone (M3 vs. M5: p's>0.05).

Discussion

The current cross-sectional study provides the first glimpse at amygdala nuclei volume apportionment in adolescents. While previous studies have examined developmental changes in the total amygdala volume across childhood and adolescence (Herting et al., 2018; Wierenga et al., 2018), the current study highlights the utility of the CIT168 to define 9 amygdala subregions in a large sample of adolescents and suggests that amygdala composition may continue to modify across the adolescent period in relation to sex. Using the newly derived in vivo CIT168 atlas, relative changes in the subregion composition of the amygdala were associated with age in males, but not females. In males, findings suggest an expansion in relative volumes of the LA and CEN, but contraction of the BLVPL and ATA subregions, accounting for between 6 to 13% of the variance in the relative composition of these regions within the amygdala.
Our findings support the hypothesis that relative volumes occupied by nuclei within the amygdala may undergo structural reformation during the adolescent years, although only in males. While MRI and the CIT168 atlas cannot decipher each of the exact 13 nuclei of the human amygdala, our findings in males are supported by the recent histology study showing that postnatal neuron numbers change in distinct nuclei, including the central, lateral, and basal nuclei, from childhood to adulthood (Avino et al., 2018). In that study, however, a sex-specific effect was not examined, as the wide age range (n=24, 2 to 48 years) neurotypical sample had very few females (n = 5) (Avino et al., 2018). Beyond nucleus-specific changes in neuron number, postnatal immunohistochemistry studies have also found a difference in immature and mature neuron concentrations among amygdala nuclei, including the lateral, central, basal, and paralaminar nucleus (Avino et al., 2018). A higher concentration of immature neurons has been reported in the paralaminar nucleus (part of the BLVPL subregion in the current study) as compared to other amygdala nuclei (Avino et al., 2018). Moreover, the number of immature neurons in the paralaminar nucleus decreases over time, whereas the mature neuron numbers of the surrounding regions continue to increase in childhood and adolescence. These data have led to the hypothesis that gradual maturation and migration of paralaminar immature neurons may contribute to the mature neuron number within the paralaminar, and/or be the source of increases in neuron number seen in other nuclei over development. If this hypothesis proves to be correct, migration and maturation of immature neurons may contribute to the re-configuration and/or refinement of the amygdala subregions and their subsequent connectivity with the cerebral cortex across adolescence. While MRI cannot assess neuron number, more research is needed to determine if decreases in the relative fraction of the BLVPL and ATA but increases in the surround LA and CEN in males may be suggestive of distinct nuclei maturation and migration patterns in amygdala development. Combining postmortem histology and MRI segmentation approaches in
developing samples is necessary to further decipher if these age and sex-specific patterns occur across development.

Furthermore, cytoarchitectural findings suggest the BLVPL of the amygdala receives afferents from both the lateral nucleus (LA) and the hippocampus (Pitkanen and Amaral, 1998). Efferents of the medial paralaminar nucleus gradually merge with the periamygdaloid cortex, often termed the “corticoamygdaloid transition area”, which further projects to the hippocampus. Moreover, the lateral nucleus (LA) receives sensory information, allowing the basolateral complex to process the information, and then send this information out of the amygdala via the central nucleus (CEN) (McDonald and Jackson, 1987; Sah et al., 2003). The CIT168 ATA region encapsulates the periamygdaloid cortex, as well as these amygdalocortical and amygdolohippocampal transition areas. Hippocampal input to the amygdala is important for contextual fear learning (Phillips and LeDoux, 1992), and given the convergence between sensory input from the LA, as well as bidirectional connectivity with the hippocampus, it has been proposed that the paralaminar and periamygdaloid cortex of the amygdala may be involved in contextual learning (deCampo and Fudge, 2013). It remains to be elucidated how age expansion for the LA, CEN, but contraction for the BLVPL and ATA, in males may map onto function. However, amygdala nuclei composition may be an additional MRI feature to explore in hopes of clarifying our understanding of amygdala structural and functional development. It may also prove useful in studying known sex differences in emotion-related behavior, brain function, and prevalence in mental health disorders that typically emerge during this time. For example, meta-analysis of 166 studies found a small, yet consistent, sex difference in positive and negative emotional expression that begins to diverge in the beginning of childhood and into adolescence (Chaplin and Aldao, 2013). Similarly, fMRI studies have reported greater brain activity in cortical regions, including visual and parietal regions, in male versus female adolescents during emotional functional MRI tasks (Cservenka et al., 2015). Resting-state fMRI studies implementing ex vivo atlases to define...
basolateral, superficial, and centromedial subregions, have also found age and sex-specific differences in amygdala functional connectivity patterns. Age and region-specific patterns were seen between the amygdala and medial prefrontal cortex, with connectivity becoming apparent at age 10 and continuing to strengthen across adolescence (Gabard-Durnam et al., 2014). In a separate study, the superficial amygdala resting-state patterns were found to be more mature in female adolescents, but basolateral amygdala connectivity patterns were more mature in male adolescents (Alarcon et al., 2015). Future studies are warranted to determine if the relative expansion of the primary input (LA) and output (CEN) subregions, but contraction of contextual and emotional learning subregions (BLVPL, ATA) in males, may relate to differences in emotional expression, greater cortical activation to emotional stimuli and/or stronger basolateral functional connectivity in males versus females during adolescence. Beyond the possible functional implications of nuclei apportionment, implementation of the CIT168 atlas to construct ROIs for other MRI modalities, including resting-state fMRI, task-based fMRI, and diffusion, may also assist in gaining greater specificity of how different amygdala nuclei functionally and structurally develop.

While this is the first study to examine amygdala nuclei volume composition in adolescents, the current study has both strengths and limitations. Other amygdala segmentation approaches are derived from post-mortem samples that are largely based on smaller samples of older male brains (Amunts et al., 2005; Saygin et al., 2017), which not only fail to capture possible developmental changes but may also be confounded by factors that influence tissue quality (Stan et al., 2006). The CIT168 atlas mitigates some of these concerns by using the high-resolution (700 micrometer) in vivo Human Connectome Project data from young adults (ages 22-35 years). Furthermore, the current study illustrates the ability to apply this newly developed CIT168 atlas to assess 9 distinct amygdala subregions in adolescents, given our similar total probabilistic and relative amygdala volumes based on our adolescent T1-weighted images and the CIT168 T1 and T2-weighted images (Tyszka and Pauli, 2016). Moreover, our hypothesis that physical growth
metrics, including body mass and pubertal development would relate to amygdala composition during adolescence was not supported. While physical characteristics of pubertal maturation did relate to BLVPL, CEN, and ATA, our current results suggest that age alone best accounts for individual differences in amygdala nuclei volume composition in males. Moreover, neither age nor pubertal status related to any of the nuclei examined in females. It is possible the lack of associations is due to our study sample. Although pubertal development scores were on average similar between the sexes in our sample (Table 2), there were fewer females that fell within the pre-pubertal and early pubertal range as compared to males in this age range of 10 to 17 years. While this is to be expected given the known sex difference in pubertal onset, with girls showing physical signs of maturation ~1-2 years prior to males (Dorn, 2006), more research is needed in younger females in order to assess if similar patterns of amygdala maturation do occur at slightly younger ages in females. Furthermore, it would also be helpful to utilize other markers that may be more accurate for capturing both puberty and obesity in children, such as pubertal hormone levels and measurements of body composition.

To summarize, we show the adolescent amygdala can be segmented into 9 subregions using the newly developed CIT168 atlas and that the relative composition of these amygdala subregions may continue to restructure in a sex-specific fashion during the adolescent window of development. By using this approach in conjunction with considering how the amygdala nuclei composition may continue to develop, future studies may be able to further explore how the amygdaloid complex may interact with distinct cortical regions, such as the prefrontal cortex, in order to modulate each other’s development and social and emotional behaviors that continue to mature during this critical period in development (Andersen and Teicher, 2008; Tottenham and Gabard-Durnam, 2017). Our approach provides a first step towards a more rigorous exploration of functional and structural connectivity development within the heterogeneous amygdala complex across adolescence.
Figure Legends

Figure 1: Probabilistic segmentation of amygdala subregions in a representative adolescent. Structural MRI and probabilistic estimates of 9 bilateral subregions shown in the A) coronal and B) sagittal view (thresholded at probabilistic value of .3 for visualization purposes).

Key: LA, lateral nucleus; BLDI, basolateral dorsal and intermediate subdivision; BLVPL, basolateral ventral and paralaminar subdivision; BM, basomedial nucleus; CMN, cortical and medial nuclei; CEN, central nucleus; AAA, anterior amygdala area; ATA, amygdala transition area; ASTA, amygdalostriatal transition area. Based on CIT168 atlas, regions of the amygdala not assigned to a specific subregion are collected into the whole AMY (other) label.
Figure 2: Overlay of CIT168 segmentation on coronal slices through entire rostral-caudal view of the amygdala in the right hemisphere for four representative subjects. A maximum likelihood label was created for each subregion of the amygdala by creating a label based on a simple competition between probabilistic labels with a thresholded probabilistic value of .3 for visualization purposes; slices (1mm) are sequential (no gap).
Figure 2-1: Outline of CIT168 segmentation on coronal slices through entire rostral-caudal view of the amygdala in the right hemisphere for four representative subjects. A maximum likelihood label was created for each subregion of the amygdala by creating a label based on a simple competition between probabilistic labels with a thresholded probabilistic value of .3 for visualization purposes; slices (1mm) are sequential (no gap).
Figure 3: Amygdala subregion volumes and relative volume fractions in adolescent males and females. A) Probabilistic volumes (mm$^3$) and B) relative volume fraction (RVF; proportional to total amygdala volume) for each of the 9 bilateral amygdala subregion ROIs.
Figure 4: Sex differences in age associations with RVF of the amygdala subregions. A) Lateral nucleus (LA), B) Basolateral ventral and paralaminar subdivision (BLVPL) and C) Central (CEN) and D) Amygdala transition area (ATA). RVF plotted by age and sex (collapsed across hemispheres); solid lines reflect GAMM predicted fit estimates and dashed lines reflect 95% confidence intervals.
## Tables

### Table 1. Amygdala Subregion ROIs

| Subregion name | CIT168 Regions |
|----------------|----------------|
| Lateral nucleus | Basolateral dorsal and intermediate subdivision | Basolateral ventral and paralaminar subdivision | Basomedial nucleus | Cortical and medical nuclei | Central nucleus | Anterior amygdala area | Amygdala transition area | Amygdala transition transition area |
| Subregion abbreviation | (LA) | (BLDI) | (BLVPL) | (BM) | (CANN) | (CEN) | (AAA) | (ATA) | (ASTA) |
| Subregion location or other common names | Surrounded ventrally and caudally by lateral ventricle, and laterally by temporal lobe white matter; primary input from neocortex | Subdivision of the basolateral nucleus; the basolateral nucleus lies medially to the lateral nucleus (LA) | Subdivision of the basolateral nucleus; the basolateral nucleus lies medially to the lateral nucleus (LA) | Ventrally bounded by BLV; BM also known as the accessory basal nucleus | Lies along dorsomedial surface of amygdaloid complex | Major output nuclei; lies dorsally and caudally within complex | Lies rostrally and caudally within complex; borders paraimygldaloid claustrum and basolateral complex | Boundary between entorhinal cortex and OVN | Lies medially and ventrally to temporal branch of anterior commissure; borders ventral putamen |
| Subregion content | LA exclusively | Merger between the basolateral nucleus’ 2 out of 3 divisions: dorsal (BDI) and intermediate (BLI) | Merger between the basolateral nucleus’ 3rd division, ventral (BLV), and the paralaminar nucleus | BM exclusively | BM exclusively | BM exclusively | BM exclusively | BM exclusively | BM exclusively |

Descriptions based on the CIT168 atlas by Tyszka JM, Pauli WM (2016)

### Table 2. Sample characteristics

#### A) Demographics of study participants

|           | All       | Female     | Male       | Difference between Male and Female |
|-----------|-----------|------------|------------|------------------------------------|
|           | N | Mean | SD | N | Mean | SD | N | Mean | SD | df | Coefficient | p-value |
| Age       | 421 | 14.13 | 1.64 | 186 | 14.00 | 1.60 | 235 | 14.22 | 1.67 | 417 | 0.37 | 0.81 |
| BMIz      | 421 | 0.50 | 0.99 | 186 | 0.47 | 0.92 | 235 | 0.52 | 1.04 | 417 | 0.42 | 0.49 |
| ICV       | 421 | 1464124 | 139299 | 186 | 1372108 | 103182 | 235 | 1536954 | 119808 | 417 | 121231 | <0.0001 |
| PDS       | 421 | 2.78 | 0.77 | 186 | 3.06 | 0.72 | 235 | 2.56 | 0.74 | 417 | -5.60E-02 | 0.93 |

#### B) Associations between predictors

|           | All       | Female     | Male       | Difference between Male and Female |
|-----------|-----------|------------|------------|------------------------------------|
|           | df | Coefficient | p-value | df | Coefficient | p-value | df | Coefficient | p-value | df | Coefficient | p-value |
| Age to ICV | 417 | 1.5E-07 | 0.84 | 184 | 2.5E-07 | 0.83 | 234 | 5.4E-08 | 0.95 | 417 | -1.4E-07 | 0.90 |
| ICV to BMIz | 417 | -9115 | 0.11 | 184 | -2855 | 0.73 | 234 | -15375 | 0.04 | 417 | -8853 | 0.27 |
| BMIz to Age | 417 | 0.03 | 0.39 | 184 | 0.05 | 0.29 | 234 | 0.0066 | 0.87 | 417 | -0.03 | 0.52 |
| PDS to ICV | 417 | 1.9E-07 | 0.57 | 184 | 3.4E-07 | 0.51 | 234 | 3.2E-08 | 0.94 | 417 | -2.2E-07 | 0.64 |
| BMIz to PDS | 417 | 0.19 | 0.004 | 184 | 0.29 | 0.002 | 234 | 0.09 | 0.33 | 417 | -0.14 | 0.13 |
Notes: P-values with significance level of less than 0.05 are bolded. Abbreviations: SD, standard deviation; BMIz, Body Mass Index z-score; PDS, Pubertal Development Scale; ICV, Intracranial Volume

**Table 3. Probabilistic amygdala subregions by sex**

A) Absolute probabilistic volume for each subregion (mm³)

| Sex | Hemisphere | LA  | Mean | CoV (%) | BLDI | Mean | CoV | BLVPL | Mean | CoV | BM  | Mean | CoV | CMN  | Mean | CoV | CEN  | Mean | CoV | AAA  | Mean | CoV | ATA  | Mean | CoV | ASTA | Mean | CoV |
|-----|------------|-----|------|---------|------|------|-----|-------|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|
| Female | Right | 332.02 | 9.99 | 201.36 | 9.87 | 121.69 | 10.92 | 115.19 | 9.85 | 171.46 | 9.00 | 48.07 | 9.95 | 62.82 | 10.34 | 92.74 | 9.95 | 69.58 | 10.50 |
| Male   | Right | 341.98 | 11.22 | 198.02 | 10.77 | 118.86 | 11.16 | 114.10 | 10.22 | 170.75 | 9.33 | 46.96 | 10.46 | 62.59 | 11.26 | 96.43 | 10.18 | 68.99 | 11.34 |

B) Relative volume fraction for each subregion (to total amygdala volume)

| Sex | Hemisphere | LA  | Mean | CoV (%) | BLDI | Mean | CoV | BLVPL | Mean | CoV | BM  | Mean | CoV | CMN  | Mean | CoV | CEN  | Mean | CoV | AAA  | Mean | CoV | ATA  | Mean | CoV | ASTA | Mean | CoV |
|-----|------------|-----|------|---------|------|------|-----|-------|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|
| Female | Right | 0.21 | 4.89 | 0.13 | 2.75 | 0.08 | 5.46 | 0.07 | 3.75 | 0.11 | 4.63 | 0.03 | 6.84 | 0.04 | 6.02 | 0.06 | 6.58 | 0.04 | 6.74 |
| Male   | Right | 0.22 | 5.27 | 0.12 | 2.84 | 0.07 | 5.32 | 0.07 | 4.00 | 0.11 | 4.49 | 0.03 | 6.32 | 0.04 | 6.63 | 0.06 | 6.80 | 0.04 | 7.23 |

Notes: Mean and coefficient of variation (CoV, mean/SD x 100%) for each subregion’s probabilistic volume in millimeters cubed (mm³) or Relative Volume Fractions (RVF) in each brain hemisphere (right and left) for both males and females. Abbreviations: See Table 1.
Table 4. GAML results for amygdala subregion RVF associations with age, sex, and age-sex interaction, controlling for hemisphere, BMI, and ICV.

|     | LA | CEN |
|-----|----|-----|
| s(age) | 0 | 3 | 3 | 1.00 | 0.50 | 3 | 0.28 | 0.18 |
| s(age*sex(male)) | 1.26 | 3 | 2.91 | 0.002 | 2.40 | 3 | 5.02 | 0.0002 |
| Intercept | 0.21 | 0.006 | 32.87 | <0.0001 | 0.03 | 0.001 | 26.30 | <0.0001 |
| Sex (male) | 0.001 | 0.0009 | 0.74 | 0.46 | -0.001 | 0.0002 | -3.36 | 0.001 |
| Hemisphere (right) | 0.01 | 0.0006 | 68.88 | <0.0001 | -0.001 | 0.0001 | -7.40 | <0.0001 |
| BMI | 0.001 | 0.0005 | 1.20 | 0.23 | 0.0001 | 0.0001 | 1.22 | 0.22 |
| ICV | 0 | 0 | -0.20 | 0.84 | 0 | 0 | 0.15 | 0.88 |

|     | BLVPL | AAA |
|-----|-------|-----|
| s(age) | 0 | 3 | 0 | 0.44 | 0.17 | 3 | 0.07 | 0.28 |
| s(age*sex(male)) | 0 | 3 | 0 | 0.33 | 0 | 3 | 0 | 0.72 |
| Intercept | 0.13 | 0.002 | 62.88 | <0.0001 | 0.04 | 0.001 | 31.41 | <0.0001 |
| Sex (male) | 0.003 | 0.0016 | 0.88 | 0.39 | 0 | 0.0002 | 0.08 | 0.94 |
| Hemisphere (right) | -0.003 | 0.0002 | -17.00 | <0.0001 | -0.0002 | 0.0001 | -1.61 | 0.11 |
| BMI | -0.0001 | 0.0002 | -0.88 | 0.38 | 0.0001 | 0.0001 | 0.92 | 0.36 |
| ICV | 0 | 0 | 1.04 | 0.30 | 0 | 0 | -2.17 | 0.03 |

|     | BLVPL | ATAPIA |
|-----|-------|-------|
| s(age) | 2.20 | 3 | 8.71 | 0.71 | 0 | 3 | 0 | 0.68 |
| s(age*sex(male)) | 0 | 3 | 0 | 0.42 | 2.17 | 3 | 16.37 | <0.0001 |
| Intercept | 0.07 | 0.002 | 32.03 | <0.0001 | 0.06 | 0.002 | 26.78 | <0.0001 |
| Sex (male) | 0.001 | 0.0003 | 1.85 | 0.07 | 0.001 | 0.0003 | 3.23 | 0.001 |
| Hemisphere (right) | -0.003 | 0.0002 | -11.22 | <0.0001 | 0.001 | 0.0002 | 6.80 | <0.0001 |
| BMI | -0.0004 | 0.0002 | -1.94 | 0.05 | 0.0004 | 0.0002 | -1.98 | 0.05 |
| ICV | 0 | 0 | 1.42 | 0.15 | 0 | 0 | -0.55 | 0.58 |

|     | BMI | ASTA |
|-----|-----|-----|
| s(age) | 0 | 3 | 0 | 0.68 | 0 | 3 | 0 | 0.50 |
| s(age*sex(male)) | 0 | 3 | 0 | 0.42 | 1.49 | 3 | 2.64 | 0.01 |
| Intercept | 0.07 | 0.002 | 40.23 | <0.0001 | 0.04 | 0.002 | 26.54 | <0.0001 |
| Sex (male) | -0.001 | 0.0002 | -2.27 | 0.02 | -0.001 | 0.0002 | -3.36 | 0.001 |
| Hemisphere (right) | -0.001 | 0.0002 | -8.03 | <0.0001 | -0.001 | 0.0002 | -3.65 | 0.003 |
| BMI | -0.0002 | 0.0001 | -1.18 | 0.24 | 0.0002 | 0.0001 | 1.23 | 0.22 |
| ICV | 0 | 0 | 2.91 | 0.004 | 0 | 0 | -0.49 | 0.63 |

|     | CMN |     |
|-----|-----|-----|
| s(age) | 0 | 3 | 0 | 0.53 | 0.11 | 0.003 | 37.88 | <0.0001 |
| s(age*sex(male)) | 0.41 | 3 | 0.21 | 0.21 | -0.0002 | 0.0004 | -0.50 | 0.62 |
| Intercept | 0.11 | 0.003 | 37.88 | <0.0001 | -0.002 | 0.0003 | -5.76 | <0.0001 |
| Sex (male) | -0.002 | 0.0002 | -0.27 | 0.79 | 0.0001 | 0.0001 | -1.01 | 0.31 |
| Hemisphere (right) | 0 | 0 | -1.01 | 0.31 |

Notes: In each model, for the parametric terms, the estimate, standard error (SE), t-value, and p-value are shown, for the smooth terms, the estimated degree of freedom (edf), reference degree of freedom (Ref.df), F-score, and p-value are shown; the Adjusted R^2 for each model is also shown. P-values of significance level less than 0.0056 bolded. Abbreviations: See Table 1.
Table 5. GAMM amygdala subregion results for age, pubertal status, and age-by-pubertal status interaction for males

| Subregion | Smooth Terms | Model Fit | Model Fit |
|-----------|--------------|-----------|-----------|
| LA        | Smooth Terms | Model Fit | Model Fit |
| Males     | Smooth Terms | Model Fit | Model Fit |
| LA        | Smooth Terms | Model Fit | Model Fit |
| BLVPL     | Smooth Terms | Model Fit | Model Fit |
| BM        | Smooth Terms | Model Fit | Model Fit |
| CMN       | Smooth Terms | Model Fit | Model Fit |
| CEN       | Smooth Terms | Model Fit | Model Fit |
| AAA       | Smooth Terms | Model Fit | Model Fit |
| ATA       | Smooth Terms | Model Fit | Model Fit |
| ASTA      | Smooth Terms | Model Fit | Model Fit |

Notes: In each model, the smooth terms, the estimated degree of freedom (edf), reference degree of freedom (Ref.df), F-score, p-value, and Adjusted R² for each model is shown; p-value < 0.0056 bolded (Bonferroni corrected). Between model comparisons include the df, AIC, log-likelihood ratio (L Ratio) and p-values < 0.05 bolded.

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### Table 6. GAMM amygdala subregion results for age, pubertal status, and age-by-pubertal status interaction for females

| LA      | Smooth Terms | F      | p-value | R2   | df | AIC  | BIC  | logLik | Test | L.Ratio | p-value |
|---------|---------------|--------|---------|------|-----|------|------|--------|------|---------|---------|
| M3      | s(age)        | 0.0386 | 0.579   | -2325.71 | 119.86 | 0.56 | 0.967 |
| M4      | s(pds)        | 0.0386 | 0.730   | -2325.71 | 119.86 | 0.56 | 0.967 |
| M5      | t(age)        | 0.0381 | 0.910   | -2318.28 | 117.14 |      |       |
|         | t(pds)        | 1.056  | 0.455   | -2275.23 | 117.14 |      |       |
|         | t(age, pds)   | 1.056  | 0.455   | -2275.23 | 117.14 |      |       |
| BLDI*   | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.186  | 1.000   | -3180.56 | 1597.28 | 0.00 | 1.000 |
| M4      | s(pds)        | 0.186  | 1.000   | -3180.56 | 1597.28 | 0.00 | 1.000 |
| M5      | t(age)        | 0.186  | 0.915   | -3172.56 | 1597.28 |      |       |
|         | t(pds)        | 1.002  | 0.966   | -3129.51 | 1597.28 |      |       |
|         | t(age, pds)   | 1.002  | 0.966   | -3129.51 | 1597.28 |      |       |
| BLVPL   | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 1.60   | 1.05    | -3035.01 | 1524.50 | 3.16 | 0.532 |
| M4      | s(pds)        | 0.766  | 1.000   | -3035.76 | 1524.88 | 2.41 | 0.662 |
| M5      | t(age)        | 0.187  | 0.290   | -3030.17 | 1526.08 |      |       |
|         | t(pds)        | 1.73   | 1.05    | -2987.12 | 1526.08 |      |       |
| BM      | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0423 | 0.530   | -3302.28 | 1658.14 | 1.46 | 0.833 |
| M4      | s(pds)        | 0.0423 | 0.530   | -3302.28 | 1658.14 | 1.46 | 0.833 |
| M5      | t(age)        | 0.0455 | 0.789   | -3295.74 | 1658.87 |      |       |
|         | t(pds)        | 1.45   | 0.229   | -3252.69 | 1658.87 |      |       |
| CMN     | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0449 | 0.530   | -2916.17 | 1645.08 | 1.98 | 0.740 |
| M4      | s(pds)        | 0.0449 | 0.530   | -2916.17 | 1645.08 | 1.98 | 0.740 |
| M5      | t(age)        | 0.0599 | 0.561   | -2910.14 | 1646.07 |      |       |
|         | t(pds)        | 2.08   | 2.12    | -2867.10 | 1646.07 |      |       |
| CEN     | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0485 | 0.424   | -3564.77 | 1789.39 | 1.89 | 0.756 |
| M4      | s(pds)        | 0.0436 | 0.476   | -3564.36 | 1789.18 | 2.30 | 0.681 |
| M5      | t(age)        | 0.0656 | 0.116   | -3558.66 | 1790.33 |      |       |
|         | t(pds)        | 2.08   | 2.25    | -3515.61 | 1790.33 |      |       |
| AAA     | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0176 | 0.268   | -3390.84 | 1702.42 | 3.90 | 0.419 |
| M4      | s(pds)        | 0.0245 | 0.709   | -3391.81 | 1702.91 | 2.93 | 0.570 |
| M5      | t(age)        | 0.0268 | 0.620   | -3386.74 | 1704.37 |      |       |
|         | t(pds)        | 2.08   | 2.25    | -3343.69 | 1704.37 |      |       |
| ATA     | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0631 | 0.594   | -3057.41 | 1535.71 | 3.65 | 0.458 |
| M4      | s(pds)        | 0.0640 | 0.729   | -3057.43 | 1535.71 | 3.64 | 0.458 |
| M5      | t(age)        | 0.0731 | 0.889   | -3053.06 | 1537.53 |      |       |
|         | t(pds)        | 1.057  | 0.057   | -3010.01 | 1537.53 |      |       |
| ASTA    | Smooth Terms  |        |         |       |     |      |      |        |      |         |         |
| M3      | s(age)        | 0.0827 | 0.619   | -3263.98 | 1638.99 | 0.06 | 1.000 |
| M4      | s(pds)        | 0.0827 | 0.838   | -3263.98 | 1638.99 | 0.06 | 1.000 |
| M5      | t(age)        | 0.0827 | 0.528   | -3256.04 | 1639.02 |      |       |
|         | t(pds)        | 1.06   | 0.811   | -3212.99 | 1639.02 |      |       |

Notes: In each model, the smooth terms, the estimated degree of freedom (edf), reference degree of freedom (Ref.df), F-score, p-value, and Adjusted R² for each model is shown; p-value < 0.0056 bolded (Bonferroni corrected). Between model comparisons include the df, AIC, log-likelihood ratio (L Ratio) and p-values < 0.05 bolded. *For model to converge, 5 knots were chosen instead of 4.
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