Associations Between Testosterone, Estradiol, and Androgen Receptor Genotype With Amygdala Subregions in Adolescents

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Title: Associations between testosterone, estradiol, and androgen receptor genotype with amygdala subregions in adolescents

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Abstract

Much is known about the development of the whole amygdala, but less is known about its structurally and functionally diverse subregions. One notable distinguishing feature is their wide range of androgen and estrogen receptor densities. Given the rise in pubertal hormones during adolescence, sex steroid levels as well as receptor sensitivity could influence age-related subregion volumes. Therefore, our goal was to evaluate the associations between the total amygdala and its subregion volumes in relation to sex hormones – estradiol and free testosterone (FT) – as a function of age and genetic differences in androgen receptor (AR) sensitivity in a sample of 297 adolescents (46% female). In males, we found small effects of FT-by-age interactions in the total amygdala, portions of the basolateral complex, and the cortical and medial nuclei (CMN), with the CMN effects being moderated by AR sensitivity. For females, small effects were seen with increased genetic AR sensitivity relating to smaller basolateral complexes. However, none of these small effects passed multiple comparisons. Future larger studies are necessary to replicate these small, yet possibly meaningful effects of FT-by-age associations and modulation by AR sensitivity on amygdala development to ultimately determine if they contribute to known sex differences in emotional neurodevelopment.

Keywords: amygdala, androgen receptor, adolescence, lateral amygdala, testosterone, estradiol

1. Introduction

The amygdala – a gray matter region – continues to increase in volume during childhood and adolescence, with larger growth seen in males as compared to females (Giedd et al. 1996, Herting et al. 2018). The amygdala may also be more sensitive to the rising levels of sex steroids that occurs across adolescence with puberty since it has a high density of androgen receptors (AR) and estradiol receptors (Simerly et al. 1990), ultimately accounting for sex differences in amygdala volumes (Lenroot and Giedd 2010). However, previous human neuroimaging studies have found mixed results linking hormone levels

with whole amygdala volumes. In males, Neufang et al. (2009) found that circulating testosterone predicted T1-weighted amygdala intensity values regardless of age. Bramen et al. (2012) found no cross-sectional association between testosterone and whole amygdala volumes, whereas in a follow-up longitudinal study of the same sample, higher levels of testosterone in males related to greater decreases in right amygdala volumes with age across adolescence (Herting et al. 2014). In contrast, longitudinally, higher levels of testosterone have also been associated with larger amygdala volumes across both hemispheres after adjusting for age (Wierenga et al. 2018). In females, testosterone predicted T1-weighted amygdala intensity values (Neufang et al. 2009), whereas testosterone levels were associated with smaller right amygdala volumes cross-sectionally (Bramen et al. 2012) or greater decreases in right amygdala volumes with age (Herting et al. 2014). These studies also found higher estradiol levels related to trend-level increases in right amygdala volumes with age (Herting et al. 2014), but did not relate to T1-weighted amygdala intensity values in females regardless of age (Neufang et al. 2009). These initial studies suggest that while sex steroids may be important to amygdala development, more work is needed to clarify the associations between rising hormone levels and differences in amygdala volumes that occur with age across adolescence.

In further clarifying the role of sex hormones and amygdala morphology throughout adolescence, it is essential to acknowledge that the amygdala is comprised of heterogeneous subregions (Sah et al. 2003). Based on cytoarchitecture and connectivity, amygdala nuclei are classified into subgroups, each of which plays functionally diverse roles. The basolateral nuclear group – which includes regions such as the lateral, basolateral and paralaminar nuclei – is the main input to the amygdala, converging sensory and prefrontal cortex information (Sah et al. 2003). The corticomedial group – which includes the medial, cortical, nucleus of the lateral olfactory tract, and periamygdaloid cortex – receives autonomic and monoaminergic brain stem inputs (Felten et al. 2016). Other subregions include the central nucleus – the major output of the amygdala – as well as transition regions between the amygdala and other limbic and reward regions. The importance of considering these distinct sets of subregions in understanding amygdala development has further been highlighted by a recent human postmortem study showing

changes in neuron numbers within the amygdala from 2 to 48 years of age primarily in males, with the basolateral group showing the most robust change (Avino et al. 2018). More recently, we utilized a novel *in vivo* amygdala segmentation technique to divide the human amygdala into 9 subregions (Tyszka and Pauli 2016) to examine associations between age and amygdala subregion volumes across adolescence (ages 10-17 years, N=408) (Campbell et al. 2021). We found a strong sex-specific age effect in amygdala subregion volumes during adolescence in basolateral and central subregions in males, but not females (Campbell et al. 2021).

While rising levels of free testosterone (FT) that occur during adolescence presumably activates androgen receptors (AR) to ultimately impact amygdala development, individual AR function may have independent and/or moderating effects. The AR gene contains a polymorphic region with variable CAG repeat numbers, which has been shown to inversely relate to AR transcriptional activity in human cell cultures (Chamberlain et al. 1994). This common polymorphism may indicate differences in AR sensitivity following androgen activation, and has more recently been found to moderate the association between testosterone and patterns of global brain volumes across human adolescence (Paus et al. 2010), as well as amygdala reactivity to facial expression in adult human males (Manuck et al. 2010). In the current study we aim to determine how rising sex hormone levels with age and genetic differences in AR sensitivity relate to amygdala volumes and its subregions in 297 adolescents (females = 46%). Our aims were to: 1) assess potential interactions of age and sex hormones – FT or estradiol – on volumes of the amygdala and its subregions; 2) determine whether AR sensitivity relates to these volumes; and 3) examine whether there is a moderating effect of AR sensitivity on the testosterone-by-age interactions. Given that sex hormone levels increase with age across adolescence as a function of puberty (Vigil et al. 2016, Herting and Sowell 2017), we elected to examine the interaction term of hormone with age to account for the potential moderating effects of age on hormone levels, given the previous research showing age-by-testosterone effects in the right amygdala (Herting et al. 2014). Even though previous human literature is mixed (Herting et al. 2014, Wierenga et al. 2018), we hypothesized that higher levels of testosterone would associate with larger amygdala volumes as a function of age given previous animal

studies (Fowler et al. 2003, Ahmed et al. 2008). Although this is the first study to examine how AR sensitivity may relate to human amygdala volumes, we believe FT effects with age are expected to be further moderated by AR sensitivity given that animal studies have shown AR density to vary widely between amygdala subregions (Simerly et al. 1990, Johnson et al. 2013). Moreover, we expect regions with high densities of AR staining (i.e., cortical and medial nuclei (CMN) and central nucleus (CEN)) may show stronger positive associations with testosterone levels, with this effect being larger when testosterone levels are higher among older adolescents. Given previous findings (Herting et al. 2014), we also expect that estradiol will have stronger association with larger amygdala volumes and this effect will be driven by older adolescent females given that estradiol is their main sex hormone.

2. Materials and Methods

2.1. Participants

Cross-sectional data was collected from 297 adolescents (n=137 / 46% females, ages 10-17) as part of an ongoing study at the Oregon Health & Science University (OHSU) under a protocol approved by the Institutional Review Board of OHSU. Each participant received a comprehensive interview to determine their eligibility, with written assent and consent obtained from each participating adolescent and at least one biological parent. To limit variation in our sample, all subjects were right-handed and free of neurological, neurodevelopmental, and/or psychological diagnoses; to remove the potential impact sibling pairs could have on our dataset, one sibling was randomly chosen from each family. Detailed exclusionary criteria explanation for this same sample can be found in the supplement of Campbell et al. (2021). We also collected socioeconomic status (SES) for each individual based on the Hollingshead Four-Factor Index (Hollingshead 1975). Outliers were also removed if they fell outside of three standard deviations. After the removal of outliers (Male: estradiol n=1, AR n=1; Female: estradiol n=2, FT n=3, AR n=3), the final sample size for hormone data was 297. Of these subjects, 197 subjects (n=89 / 45% females) were included for AR genotype modeling given that only certain subjects consented to the option of future genotyping of their biological samples.

2.2 Sex hormone levels

All subjects had four millimeters of their blood drawn by venipuncture between 7:00 and 10:00 AM at the Oregon Clinical and Translational Research Institute. For females that had begun menarche – given the cyclicity of their menstrual cycle – their study visits, including blood draw, was collected during their follicular cycle (days 1-10 after the first day of their period) to minimize variance. Females who had not yet started menarche, were brought in at any point within the month. The Coat-A-Count radioimmunoassay (Diagnostic Product Corp., Los Angeles, CA) was used to assess total circulating testosterone; coefficients of variation (CVs) for the intra- and interassay were 7.0% and 7.4%, respectively at the lower detection level of >10ng/dL. For total estradiol, the DSL-4800 Ultra-sensitive Estradiol Radioimmunoassay Kit (Beckman Coulter (formerly DSL), Fullerton, CA) was used; CV for the intra- and interassay were 7.4% and 12.6%, respectively, at the lower detection level of >2.2pg/mL. For sex hormones binding globulin (SHBG), a chemiluminescent immunometric assay (Immulite/Immulite 1000 systems, PILKSH-10, 2006-12-29) was used; CV for the intra- and interassay were 6.9% and 13.0%, respectively, at the lower detection level of >4.5nmol/L and 6.0nmol/L, respectively. SHBG and total circulating testosterone were used to calculate free testosterone (FT) based on the Vermeulen equation (Vermeulen et al. 1999) and using an albumin constant of 4.3 g/dL; and calculations were confirmed using freely available software [<http://www.issam.ch/freetesto.htm>]. Free testosterone is the amount of testosterone that is unbound and is a better estimate of bioavailable testosterone that may ultimately bind to ARs (Vermeersch et al. 2010).

2.3 Androgen Receptor (AR) Genotype

Polymerase chain reaction (PCR) was performed on the blood sample to calculate the number of CAG repeats on the androgen receptor gene to quantify the transcriptional activity of the AR (Chamberlain et al. 1994). Primers (forward [5'- NED- GTGCGCGAAGTGATCCAGAA- 3'] and reverse [5'- TAGCCTGTGGGGCCTCTACG- 3']) were used to amplify the AR (CAG)_n repeat

(Ackerman et al. 2010). The Applied Biosystems 3130XL capillary DNA sequence analysis system was used to separate the PCR products and were genotyped by the GENEMAPPER software v4.0 (Applied Biosystems, Foster City, California). The transcriptional activity of the AR gene has been shown to have an inverse linear relationship to its activity and the length of the CAG repeats present (Chamberlain et al. 1994); therefore, the higher the number of CAG repeats, the lower the trans-activation of the AR (i.e., sensitivity). Given that the androgen receptor gene is X-linked, we created a new variable that was the biallelic mean between the two CAG repeats lengths present on both alleles for females (Shah et al. 2008). For males, the single number of CAG repeats present on the allele on the X chromosome was utilized.

2.4 MRI Data Collection and Preprocessing

MRI acquisition, preprocessing and quality control are described in detail in Campbell et al. (2021). Briefly, whole-brain T1-weighted MR images were acquired using a 3 Tesla system equipped with a 12-channel head-only receive coil (Magnetom Tim Trio, Siemens Medical Solutions, Erlangen, Germany) with the following sequence parameters: TR = 2300ms, TE = 3.58ms, TI = 900ms, flip angle = 10°, 256x240 matrix, voxel size = 1 mm x 1 mm x 1.1 mm. Each raw image was visually quality checked for motion according to the methods outlined in Backhausen et al. (2016) by A.F.M. The CIT168 probabilistic amygdala atlas (Tyszka and Pauli 2016) was registered to individual T1w structural images with the Advanced Normalization Tools (ANTs, Version 2.1.0.post691-g9bc18) (Avants et al. 2011) resulting in a probabilistic labeling of the left and right amygdala and nine amygdaloid complex subregions, including four basolateral regions [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)], superficial/corticomedial regions [cortical and medial nuclei (CMN); amygdala transition areas (ATA)], as well as the central nucleus (CEN), amygdalostratial transition area (ASTA), and anterior amygdala area (AAA). Each segmentation was visually quality checked (A.F.M.). Construction, validation, comparison with other atlases, and individual volume

distributions are described in detail in (Tyszka and Pauli 2016). Briefly, to estimate each subregion volume, each voxel in the entire brain was weighted by the probability that it belonged to that particular region of interest (ROI); this methodology differs from a deterministic model in that a deterministic model chooses an arbitrary threshold as a cut-off (typically $p > 0.5$), which does not allow for the uncertainty inherent in each voxel estimation. Subregion definitions and descriptions are provided in Tyszka and Pauli (2016) and Campbell et al. (2021). The registration and volumetric approach used here provides a robust segmentation of the subregions with a contrast-to-noise ratio (CNR) greater than 1.0 (see Tyszka and Pauli (2016) Supplementary Data). Subjects with CNR less than 1.0 were excluded from subsequent analysis (Campbell et al. 2021), including 6 females and 2 males. Mean CNR for included females ($n = 137$) was 1.37 and 1.42, and for males ($n = 160$) was 1.37 and 1.43, in the left and right hemisphere, respectively. While in our previous research we had examined both the absolute probabilistic volumes as well as the proportional effects of each subregion in relation to the total amygdala (i.e., relative volume fraction), we found similar patterns with age for both outcomes for a number of amygdala subregions (Campbell et al. 2021). Thus, in the current study, we decided to focus our analyses on the absolute probabilistic volumes of the amygdala and each subregion given the complexity of our modeling and the already large number of a priori planned analyses for the current sample size.

2.5 Statistical Analysis

All data was analyzed in R (version 4.0.2). The correlation matrix of our predictors (age, free testosterone, estradiol) and confounders (ICV, SES, menstruation status) is provided in **Supplemental Figures B.1 and B.2**. Prior to analyses, a square root transformation was performed on FT values to normalize a right skewed distribution. We examined sex hormone levels and genetic AR sensitivity associations with amygdala volumes in each sex separately in order to account for the distinct developmental trajectories seen in adolescent males and females (Lenroot and Giedd 2010) and sex-specific differences in the rise and levels of sex steroids that occur during puberty (Lauretta et al. 2018).

In an initial analysis we explored potential sex hormone (i.e., either FT or estradiol) by age interactions in each hemisphere, as well as possible main effects of sex hormone levels on amygdala volumes across both hemispheres. We briefly describe this process below; a full description of model selection is provided in **Supplemental Figure B.3**. We implemented a Generalized Additive Mixed Model (GAMM) using the *mgcv* package (version 1.8-31 in R version 4.0.2, R Core Team, 2020) to account for the nonlinearity of neurodevelopment seen during adolescent, especially in the amygdala (Herting et al. 2018, Campbell et al. 2021). Moreover, GAMMs allow the predictor function to be calculated during model estimation without an *a priori* assumption of association, as compared to linear mixed effects models. Hemisphere was modeled as a nested factor to account for the greater similarity within an individual's two hemisphere versus between subjects. Therefore, in each of these models the intercept (U_i) for each subject, i , was included as a random effect, with $Volume_{ij}$ representing the probabilistic total volume of one of the 9 subregions for each subject, i , in either the right or left hemisphere, j . All smooth terms – denoted with an 's' preceding the term in the models below – were cubic splines with four evenly spaced knots to constrain our prediction to reduce the likelihood of overfitting our model. Using this GAMM approach, our first set of models examined a potential interaction between FT and age with the total amygdala and 9 subregions independently, while accounting for potential interaction with hemisphere and covarying for ICV, SES, and menstruation (in females only) (Model 1a). The same modeling approach was also utilized for estradiol (Model 2a). If there was a significant ($p \leq 0.05$) interaction term of the sex steroid hormone – free testosterone or estradiol – and age with hemisphere, an ANOVA was conducted between this model and a main effects model without the interaction term to test whether the interaction term including hemisphere significantly improved the model's fit. Next, if models for any given region lacked significant interactions prior to correction for False Discovery Rate (FDR) with hemisphere (i.e., no difference in the shape of the association between hemispheres were found) or the ANOVA proved that the interaction term did not

significantly improve the model fit, the interaction term with hemisphere was dropped to examine the effect of FT-by-Age (Model 1b) or Estradiol-by-Age (Model 2b), while controlling for hemisphere.

Model 1a: $Volume_{ij}$

$$\begin{aligned} &= \beta_0 + s_1(Age_i, by: Hemisphere_{ij}) + s_2(FT_i, by: Hemisphere_{ij}) \\ &+ s_3(Age_i, FT_i, by: Hemisphere_{ij}) + \beta_1 ICV_{ij} + \beta_2 SES_i + \beta_3 Menstruation_i \\ &+ \beta_4 Hemisphere_i + U_i + \varepsilon_{ij} \end{aligned}$$

Model 1b: $Volume_{ij}$

$$\begin{aligned} &= \beta_0 + s_1(Age_i) + s_2(FT_i) + s_3(Age_i, FT_i) + \beta_1 ICV_{ij} + \beta_2 SES_i \\ &+ \beta_3 Menstruation_i + \beta_4 Hemisphere_i + U_i + \varepsilon_{ij} \end{aligned}$$

Model 2a: $Volume_{ij}$

$$\begin{aligned} &= \beta_0 + s_1(Age_i, by: Hemisphere_{ij}) + s_2(Estradiol_i, by: Hemisphere_{ij}) \\ &+ s_3(Age_i, Estradiol_i, by: Hemisphere_{ij}) + \beta_1 ICV_{ij} + \beta_2 SES_i + \beta_3 Menstruation_i \\ &+ \beta_4 Hemisphere_i + U_i + \varepsilon_{ij} \end{aligned}$$

Model 2b: $Volume_{ij}$

$$\begin{aligned} &= \beta_0 + s_1(Age_i) + s_2(Est_i) + s_3(Age_i, Est_i) + \beta_1 ICV_{ij} + \beta_2 SES_i \\ &+ \beta_3 Menstruation_i + \beta_4 Hemisphere_i + U_i + \varepsilon_{ij} \end{aligned}$$

Next, we wanted to see whether genetic AR sensitivity had a main effect on total probabilistic volumes, as well as volumes for each subregion, again, adjusting for ICV, SES, age, and menstruation status (for females). An identical GAMM modeling approach was used for this analysis, with initial models accounting for potential hemispheric differences (Model 3a), whereas a lack a significant interaction of AR with hemisphere resulted in dropping the interaction term to examine the main effect of AR across both hemispheres (Model 3b).

Model 3a: *Probabilist Volume_{ij}*

$$= \beta_0 + s_1(AR_i, by: Hemisphere_i) + \beta_1ICV_{ij} + \beta_2SES_i + \beta_3Age_i \\ + \beta_4Menstruation_i + \beta_5Hemisphere_i + U_i + \varepsilon_{ij}$$

Model 3b: *Probabilist Volume_{ij}*

$$= \beta_0 + s_1(AR_i) + \beta_1ICV_{ij} + \beta_2SES_i + \beta_3Age_i + \beta_4Hemisphere_i + U_i + \varepsilon_{ij}$$

Lastly, we aimed to explore if genetic AR sensitivity moderated the effects of FT on amygdala volumes. For regions that were either significant for FT (Models 1a/1b) or AR (Models 3a/3b), follow-up analyses were conducted to examine the interaction of FT and AR (Model 4a/b). For regions that were found to have a hemispheric difference, the significant hemisphere was examined exclusively for an FT-by-AR interaction. To allow for better interpretability and plotting purposes, we categorized AR repeat length into low- and high-repeat length groups by conducting a median split ($\overline{AR}_{females} = 22.11$, $\overline{AR}_{males} = 22.48$) (Perrin et al. 2008). Again, ICV, SES, and menstruation (only females) were added as covariates; the models were:

Model 4a: *Probabilist Volume_i*

$$= \beta_0 + s_1(FT_i, by: AR_cat_i) + s_2(Age_i, by: AR_cat_i) + s_3(FT_i, Age_i, by: AR_cat_i) \\ + \beta_1ICV_{ij} + \beta_2SES_i + \beta_3Menstruation_i + \beta_4AR_cat_i + U_i + \varepsilon_{ij}$$

Model 4b: *Probabilist Volume_i*

$$= \beta_0 + s_1(FT_i, by: AR_cat_i) + s_2(Age_i, by: AR_cat_i) \\ + s_3(FT_i, Age_i, by: AR_cat_i) + \beta_1ICV_{ij} + \beta_2SES_i + \beta_3Hemisphere_i \\ + \beta_4Menstruation_i + \beta_5AR_cat_i + U_i + \varepsilon_{ij}$$

Age, free testosterone (FT), estradiol, and AR repeat length were all centered on the mean in each sex separately to allow for easier interpretation of the coefficient estimates. Coefficients of determination (R^2) were used as a proxy for effect estimates demonstrating the percentage of variation explained by the whole model on the outcome of each volume (Nakagawa and Cuthill 2007). Given high correlations seen between age and FT ($r > 0.7$) in males (**Supplemental Figure B.2**), a variance inflation factor (VIF) was

assessed in males for the continuous terms in our models (i.e., Age, FT, ICV, and SES) in both the full sample and genotype subsample (Naimi et al. 2013). A VIF above 4 shows moderate collinearity with above 10 values displaying high collinearity; all our values were below 3, indicating low collinearity (Dormann et al. 2012). This test shows that despite high correlations between FT and age in males, multicollinearity was not an issue in these models. All results were controlled for multiple comparisons with False Discovery Rate (FDR) at $\alpha < 0.05$ (Benjamini and Hochberg 1995); the multiple comparisons were conducted on p-value of each predictor separately for the total amygdala and each of the subregions (i.e., 10 comparisons per model type). For each term in our model, the partial ω^2 along with its 90% confidence interval was calculated to represent each terms effect size, with ≥ 0.01 and ≥ 0.06 representing small and medium effects, respectively (Field 2013).

3. Results

Descriptives of each sex in the complete hormone dataset and the genotype subset are presented in **Table A.1** for males ($n = 160$) and females ($n = 137$). For males and females, the mean estradiol and free testosterone levels fell within existing reference values of children ages 7 to 18 years (Elmlinger et al. 2002, Khairullah et al. 2014). The subsamples used to test the main and interacting effect of androgen receptor sensitivity (males: $n = 108$; females: $n = 89$) did not significantly differ with the main sample in terms of the aforementioned descriptive statistics and covariates ($p > 0.05$; $\omega^2 < 0.1$, Cramér's $V < 0.1$); the full sample and the subsample also did not statistically differ based on an ANOVA ($p > 0.05$) in terms of the amygdala volume and each of the subnuclei volumes in either hemisphere. When comparing the high and low sensitivity groups, for males, there was a significant difference between ICV with the high AR sensitivity group having larger ICV values based on the ANOVA, but the effect was small ($\omega^2 = 0.0452$) (**Supplemental Table B.1**). For females, no significant differences were seen between the high and low AR sensitivity groups (**Supplemental Table B.2**).

Table A.1: Female and Male Demographics

	Females				Males			
	Complete Dataset (N=137)	Genotype Subset (N=89)	ω^2 (CI)	p value	Complete Dataset (N=160)	Genotype Subset (N=108)	ω^2 (CI)	p value
Age (yrs)			0.0029 (-0.0044, 0.0325)	0.200			0.001 (-0.0037, 0.0243)	0.263
Mean (SD)	14.077 (1.615)	14.351 (1.492)			14.057 (1.647)	14.281 (1.534)		
Range	10.270 – 16.879	10.270 – 16.879			10.170 – 16.996	10.170 – 16.996		
PDS			-0.0016 (-0.0044, 0.0214)	0.427			-0.0011 (-0.0037, 0.0189)	0.400
Mean (SD)	3.106 (0.706)	3.181 (0.671)			2.511 (0.729)	2.585 (0.682)		
Range	1.200 – 4.000	1.200 – 4.000			1.000 – 4.000	1.200 – 4.000		
Estradiol (pg/mL)			0.0042 (-0.0044, 0.0354)	0.163			0.002 (-0.0037, 0.0266)	0.217
Mean (SD)	28.015 (14.534)	30.747 (14.012)			18.414 (10.112)	19.986 (10.312)		
Range	4.200 – 85.000	4.200 – 85.000			4.380 – 58.100	4.380 – 58.100		
FT (nmol/L)			-0.0039 (-0.0044, 0.0107)	0.738			-3e-04 (-0.0037, 0.0212)	0.337
Mean (SD)	0.016 (0.008)	0.017 (0.008)			0.216 (0.160)	0.235 (0.159)		
Range	0.001 – 0.041	0.001 – 0.038			0.002 – 0.746	0.005 – 0.746		
ICV (mm³)			-0.0044 (-0.0044, -0.0044)	0.972			-0.0034 (-0.0037, 0.0076)	0.778
Mean (SD)	1380673 (106504)	1381177 (102988)			1540868 (121139)	1536682 (116469)		
Range	1124652 – 1655343	1127303 – 1620382			1275770- 1955787	1275770- 1955787		
Hollingshead SES			-0.0035 (-0.0044, 0.0147)	0.644			-0.0037 (-0.0037, 0.0031)	0.876
Mean (SD)	28.343 (13.779)	29.213 (13.919)			28.250 (12.831)	28.500 (12.774)		
Range	11.000 – 66.000	11.000 – 66.000			11.000 – 65.000	11.000 – 65.000		
			Cramér's V	p value				
Menses			0.0317	0.767				
No	36 (26.3%)	21 (23.6%)						
Yes	101 (73.7%)	68 (76.4%)						

Omega-squared (ω^2) represent an effect size estimate (i.e., degree of association between groups); values of 0.01 represent a small effect size. P-value represents a two-sampled t-test between the hormone dataset and the genotype subset in each sex separately. For Menses, since it is categorical, a chi-squared test was performed; Cramér's V was used to assess the effect size between the categorical variables, a value of 0.1 represents a small effect size.

Abbreviations: PDS: pubertal development status; SD: standard deviation; FT: free testosterone; ICV: intracranial volume; SES: socioeconomic status; Menses: menstruation status.

3.1 Sex Hormones and Amygdala Volumes

3.1.1 Males

Results of model 1a in males showed significant interaction terms of Age-by-FT in the right hemisphere for the total amygdala, as well as the LA, BLDI, BM, and CMN; though, the interaction with hemisphere did not significantly improve the model ($p > 0.05$). Therefore, the interaction terms were dropped for all subregions. No region passed FDR correction, but four regions – Total Amygdala, BLDI,

BLVPL, and CMN – showed trending levels ($p_{FDR} \leq 0.10$) for the FT-by-Age interaction; these regions also showed small effect sizes, with small effects sizes also seen for the BM and ASTA ($\omega^2 = [0.0108-0.0258]$) (**Supplemental Figure B.3**). Specifically, the total amygdala had a negative association with age across 10 to 17 years at lower levels of FT, whereas at higher levels of FT no association was seen between the ages of 12-17 years; the same pattern was demonstrated in the BLDI. For the BLVPL, there was similar association as the total amygdala and BLDI, but higher levels of FT only decreased the negative association seen at lower levels of FT. For the CMN, lower levels of FT had a positive association with age, while higher levels of FT, showed no association (**Supplemental Figure B.4**). Results for model 2a revealed a significant interaction term for the left Age-by-Estradiol term in the BLVPL but this interaction did not significantly improve the model fit. Therefore, no hemisphere interaction was observed for Age-by-Estradiol in males. For model 2b, no regions showed significant or trending FDR-corrected p-values and neither were there any notable effect sizes (**Supplemental Table B.4**).

3.1.2 Females:

Results of model 1a in females revealed no significant interaction of Age-by-FT with hemisphere. Follow-up analysis removing the interaction term for hemisphere similarly showed no significant or trending results for the FDR-corrected p-values, though the LA did show a small effect for the main effect of FT ($\omega^2 = 0.0279$) (**Supplemental Table B.5**). Again, no hemispheric effects were seen in females for the Age-by-Estradiol model and removing the hemisphere interaction term produced null results as well with no notable effect sizes in any of the regions (**Supplemental Table B.6**).

3.2 Androgen Receptor (AR) Sensitivity and Amygdala Volumes

3.2.1 Males:

There was a significant hemisphere interaction with AR sensitivity in the BLDI and ASTA, but the hemispheric interaction did not improve the model fit. Therefore, there were no hemispheric effects of AR sensitivity in males, and the model looking at the main effect of AR sensitivity and hemisphere as a covariate was used as the final model. The BLVPL and ATA showed trending FDR-corrected p-values

for AR sensitivity ($p \leq 0.10$) with small effect sizes ($\omega^2 = 0.03-0.033$) (**Supplemental Table B.7**). For both the BLVPL and ATA, the relationship showed higher sensitivity (i.e., lower AR CAG repeat length) associate with smaller volumes with an increase and eventually leveling of volume with lower sensitivity (**Supplemental Figure B.4**).

3.2.2 Females:

In females, a more widespread effect was seen between AR sensitivity and amygdala volumes. Initially when examining the effect of hemisphere with AR sensitivity in females, the total amygdala, LA, BLDI, BLVPL, and BM showed significant interact terms; though follow-up analysis indicated that the hemisphere interaction did improve the model fit. Therefore, no hemispheric effects were seen and the model examining the main effect of AR sensitivity was used as the final model. Results indicated an FDR-corrected trending effect in the total amygdala, LA, BLDI, BLVPL, and BM ($\omega^2 = 0.01491-0.037$) (**Table A.2**). All results showed the same association, with smaller volumes associating with higher AR sensitivity (i.e., smaller AR CAG Repeat Length) and larger volumes associating with low AR sensitivity (**Figure A.1**).

Table A.2: AR Sensitivity & Amygdala Volumes in Females

FEMALES - AR Sensitivity					
Final Model	Smooth Terms	edf	F	fdr p-value	partial ω^2 (CI)
Model 3b	Total Amygdala (R-squared = 0.39)				
	AR mean	1.018	5.141	0.070	0.0238 (-0.0038, 0.0773) ‡
Model 3b	LA (R-squared = 0.31)				
	AR mean	1.014	6.430	0.060	0.0308 (-0.0014, 0.088) ‡
Model 3b	BLDI (R-squared = 0.33)				
	AR mean	1.028	7.476	0.060	0.037 (0.001, 0.0969) ‡
Model 3b	BLVPL (R-squared = 0.30)				
	AR mean	1.017	4.879	0.070	0.0223 (-0.0043, 0.075) ‡
Model 3b	BM (R-squared = 0.36)				
	AR mean	1.014	4.316	0.077	0.0191 (-0.0052, 0.0698) ‡
Model 3b	CMN (R-squared = 0.30)				
	AR mean	1.046	1.747	0.304	0.0045 (-0.0061, 0.0436)
Model 3b	ATA (R-squared = 0.28)				
	AR mean	1.064	1.423	0.304	0.0026 (-0.0062, 0.0395)
Model 3b	CEN (R-squared = 0.27)				
	AR mean	1.019	0.194	0.662	-0.0048 (-0.0059, 0.0163)
Model 3b	AAA (R-squared = 0.17)				
	AR mean	1.005	1.049	0.341	3e-04 (-0.0058, 0.0342)
Model 3b	ASTA (R-squared = 0.32)				
	AR mean	2.548	1.182	0.304	0.0027 (-0.0149, 0.0373)

AR predictor represents average number of biallelic CAG repeats. Smooth term results for final model for each subregion; adjusted R-squared presented for full model beside subregion name. Partial ω^2 with 90% CI.

Abbreviations: edf, estimated degree of freedom; F, F-score; fdr, FDR corrected p-value; CI, confidence interval; AR, androgen receptor; Hem, hemisphere; 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, amygdalostratial transition area.

‡ $\omega^2 \geq 0.01$ (small effect)

3.3 Androgen Receptor (AR) Sensitivity and Free Testosterone Interactions

3.3.1 Males:

The volumes of the Total Amygdala, BLDI, BLVPL, CMN, and ATA were examined given previous results from FT (Model 1b) and/or AR (Model 3b) models; these models showed significant p-values ($p \leq 0.05$) and trending FDR-corrected p-values ($p \leq 0.10$). In the Total Amygdala, there was a small effect of FT for individuals with low AR sensitivity ($\omega^2 = 0.013$) (**Table A.3**). A medium effect for FT-by-Age with low AR sensitivity was seen in the CMN ($\omega^2 = 0.087$), passing FDR correction (**Table A.3**). For the BLVPL, there was a small effect for FT-by-Age with high AR sensitivity ($\omega^2 = 0.022$) (**Table A.3**). For the Total Amygdala, a positive association with FT was seen in the subjects with low AR sensitivity (**Supplemental Figure B.4**). For the BLVPL, there little to no association of FT with age at

low levels of FT in individuals with high AR sensitivity, but with higher levels of FT, there was a positive association of FT with age (**Figure A.2**). Contrastingly, there was an opposite effect in the CMN, where lower levels of FT created a steep positive association within individuals with low AR sensitivity and individuals with high levels of FT showed a negative association (**Figure A.2**). For the total amygdala and ATA, there was an overall negative relationship between testosterone and age in the low AR sensitivity group (**Figure A.2**).

Table A.3: AR Sensitivity & Free Testosterone Interactions in Males

MALES - AR Sensitivity & Free Testosterone					
Final Model	Smooth Terms	edf	F	fdr p-value	partial ω^2 (CI)
Model 4b	Total Amygdala (R-squared = 0.38)				
	FT:AR Low Sensitivity	1.814	2.526	0.175	0.0132 (-0.0089, 0.0527) ‡
	FT:AR High Sensitivity	1.696	0.451	0.797	-0.0045 (-0.0083, 0.0159)
	FT-by-Age:AR Low Sensitivity	1.084	2.575	0.255	0.0082 (-0.0053, 0.0456)
	FT-by-Age:AR High Sensitivity	1.002	0.355	0.566	-0.0032 (-0.0049, 0.0187)
Model 4b	BLDI (R-squared = 0.29)				
	FT:AR Low Sensitivity	1.031	2.342	0.175	0.0067 (-0.005, 0.0426)
	FT:AR High Sensitivity	1.003	0.072	0.814	-0.0045 (-0.0049, 0.0093)
	FT-by-Age:AR Low Sensitivity	1.006	1.621	0.255	0.003 (-0.0049, 0.0352)
	FT-by-Age:AR High Sensitivity	1.583	0.964	0.372	-3e-04 (-0.0077, 0.0272)
Model 4b	BLVPL (R-squared = 0.28)				
	FT:AR Low Sensitivity	1.007	1.841	0.175	0.0041 (-0.0049, 0.0391)
	FT:AR High Sensitivity	1.000	0.055	0.814	-0.0046 (-0.0049, 0.008)
	FT-by-Age:AR Low Sensitivity	1.001	0.060	0.807	-0.0046 (-0.0049, 0.0084)
	FT-by-Age:AR High Sensitivity	1.001	5.534	0.098	0.0215 (-0.0026, 0.0678) ‡
Model 4b	CMN (R-squared = 0.44)				
	FT:AR Low Sensitivity	2.159	2.381	0.175	0.0144 (-0.0107, 0.054) ‡
	FT:AR High Sensitivity	1.881	1.975	0.468	0.0089 (-0.0093, 0.0454)
	FT-by-Age:AR Low Sensitivity	2.823	7.886	0.002 *	0.0866 (0.0231, 0.1496) ‡‡
	FT-by-Age:AR High Sensitivity	1.007	0.328	0.566	-0.0033 (-0.005, 0.0183)
Model 4b	ATA (R-squared = 0.32)				
	FT:AR Low Sensitivity	1.000	2.123	0.175	0.0054 (-0.0049, 0.0404)
	FT:AR High Sensitivity	1.705	0.881	0.662	-0.001 (-0.0084, 0.0257)
	FT-by-Age:AR Low Sensitivity	1.116	1.876	0.255	0.0047 (-0.0055, 0.0388)
	FT-by-Age:AR High Sensitivity	1.000	2.595	0.272	0.0077 (-0.0049, 0.0448)

Smooth term results for final model for each subregion; adjusted R-squared presented for full model beside subregion name; partial ω^2 with 90% CI.

Abbreviations: edf, estimated degree of freedom; F, F-score; fdr, FDR corrected p-value; CI, confidence interval; FT, free testosterone; Hem, hemisphere; LA, lateral nucleus; BLDI, basolateral dorsal and intermediate subdivision; BLVPL, basolateral ventral and paralamina subdivision; BM, basomedial nucleus; CMN, cortical and medial nuclei; CEN, central nucleus; AAA, anterior amygdala area; ATA, amygdala transition area; ASTA, amygdalostratial transition area.

* FDR p-value ≤ 0.05 (significant)

‡ $\omega^2 \geq 0.01$ (small effect)

‡‡ $\omega^2 \geq 0.06$ (medium effect)

3.3.2 Females:

The volumes of the Total Amygdala, LA, BLDI, BLVPL and BM were examined given the previous results of the AR (Model 3b) model which showed significant p-values ($p \leq 0.05$) and trending FDR-corrected p-values ($p \leq 0.10$). When examining the effect FT-by-Age in high and low AR sensitivity groups, there were no significant associations. There was a small FT effect seen for the LA in the low AR sensitivity group ($\omega^2 = 0.0126$) (**Supplemental Table B.8**).

4. Discussion

This is the first study to investigate whether genetic differences in AR sensitivity may independently or jointly interact with hormone levels and age in predicting amygdala and its subregion volumes during adolescence. Our results display small to medium-sized effects for the interactions of testosterone, age, and AR polymorphism on amygdala volumes in males, while in females we find genetic differences in AR polymorphisms are more influential. Although only one of our findings passed multiple comparison correction, small to moderate effects have been widely reported in both the fields of psychology and neuroimaging (Meyer et al. 2001, Miller et al. 2016), suggesting our findings may still provide meaningful information regarding the role of testosterone and genetic differences in AR sensitivity in age-related patterns of amygdala subregion volumes. Moreover, our findings are congruent with previous literature showing gonadal hormones, especially testosterone, influences various aspects of emotional behavior and associated amygdala circuitry (Volman et al. 2011, McHenry et al. 2014). Below, we outline when and how these patterns may emerge in context of other known patterns of sex differences across adolescence and discuss how future studies with larger sample sizes are needed to replicate these small to moderate, yet potentially meaningful effects sizes.

Sex steroids influence brain structure and function, with prenatal testosterone thought to have long-lasting organizational effects, whereas increases in testosterone during puberty affects both brain organization and activation (Juraska et al. 2013). These processes have been thought to contribute to some of the key sex differences noted between males and females (Kaczurkin et al. 2019), including total

amygdala volume development and even potential differential risk for psychopathology (McHenry et al. 2014). Although the amygdala is thought to be especially sensitive to sex steroids, there are various mechanisms by which testosterone could ultimately exert notable sex differences in amygdala development. This includes not only the level of circulating bioavailable testosterone, but also potential AR sensitivity differences. The current study suggests sex-differences seem to exist based on which of these two mechanisms may be most important to amygdala subregion volumes when assessed cross-sectionally in adolescents. Our current findings suggest that circulating levels of bioavailable testosterone may relate to portions of the basolateral complex (BLDI and BLVPL) and the cortical and medial nuclei (CMN) in male adolescents. Given small free testosterone-by-age interaction effects noted in these regions, our findings suggest that age-related decreases seen in the basolateral complex are less apparent in those with higher testosterone levels in mid- to late-adolescence. Previous work displayed larger amygdala volumes with higher levels of testosterone (Wierenga et al. 2018); our work shows that these larger volumes may in fact be due to smaller negative associations instead of linear positive associations seen with higher FT levels. Interestingly, we find that previously published hypotheses of testosterone associating with a larger amygdala may be more nuanced since there was a trend-level and significant moderation of AR sensitivity on circulating testosterone and age in the BLVPL and CMN, respectively. However, with the current cross-sectional nature of the data, it remains to be determined if the associations between AR and amygdala volumes are specific to testosterone's actions on amygdala ARs during the adolescent period. The associations between AR and amygdala volumes reported here may also stem from testosterone – or other androgens with weaker receptor affinity (i.e., Dehydroepiandrosterone) – actions on amygdala ARs at other developmentally sensitive time periods (i.e., in utero or mini-puberty).

In contrast, amygdala subregion volumes in females were not associated with current bioavailable testosterone, but rather, lower levels of AR sensitivity (i.e., more CAG repeats) was related to larger volumes; granted these effects did not pass FDR-correction. Genetic differences in AR sensitivity were

associated bilaterally in the Total Amygdala, LA, BLDI, BLVPL, and BM in females, which again collectively represent the larger basolateral complex. Though these regions only showed small effect sizes and did not pass multiple comparison correction, they encompass a large portion of the amygdala bilaterally, with less AR sensitivity linked to larger volumes in females. Taken together, these findings suggest current bioavailable testosterone in males and AR sensitivity in both males and females are modestly linked to amygdala volumes in adolescents. This may be partially explained by differing levels of AR, aromatase expression, or possibly both, between amygdala subregions. Based on the rat *in-situ* hybridization study of Simerly et al. (1990), a high density of neurons labeled with ARs lie more medial within the amygdala in both sexes. In addition, aromatase has been shown to facilitate spine synapse density in the basolateral nucleus in mice in a sex-specific fashion (Bender et al. 2017). Supporting our initial hypothesis that regions with high AR would show the greatest association with subregion volumes, we found a statistically significant medium effect of an age and testosterone interaction moderated by AR genotype in males in the CMN – a region with the highest staining of AR in the amygdala based on a rat *in situ* study (Simerly et al. 1990). Specifically, for males with low AR sensitivity, there was a positive association of age and CMN volumes seen at lower levels of FT with age, while a negative association of age and volumes was seen at higher levels of FT. The complexity of these relationships become increasingly clear when looking at the low and high AR sensitivity at group level as compared to the entire sample, suggesting the importance of AR genotype in testosterone's effects on CMN volumes with age in adolescent males. In contrast to our hypothesis that regions with potential high AR would show the strongest relationships (i.e., CMN, CEN, and ATA), we also found volumes of the basolateral complex to associate with AR sensitivity in both sexes at trend-level significance after multiple comparison correction. Some of our original hypotheses may have been incorrect given that known patterns of AR density in the amygdala has only been established in rats (Simerly et al. 1990), which could poorly translate to humans. While these results are only trend-level, they do collocate to the basolateral complex, suggesting that this region may be especially susceptible to FT and AR effects given its anatomical connections and possible heightened plasticity during development. Specifically, the basolateral complex

bidirectionally connects with the prefrontal cortex (Sah et al. 2003), a region highly involved in regulation and maintenance of not only emotional processing, but also memory consolidation and decision making (Whittle et al. 2008, Preston and Eichenbaum 2013, Domenech and Koechlin 2015). Previous research has found that the prefrontal cortex undergoes more cortical thinning with increased AR sensitivity during adolescence in both sexes (Raznahan et al. 2010). Thus, it is feasible that AR sensitivity may be important to structural remodeling of fronto-amygdala circuitry during adolescence, so that individuals with lower AR sensitivity undergo differential patterns of pruning of the frontal cortex resulting in larger basolateral complex volumes. Previous postmortem work has also found that portions of the basolateral complex display the most robust neuronal changes from childhood to adulthood in humans (Avino et al. 2018), suggesting this area may be more malleable as compared to other amygdala subregions.

As previously mentioned, the amygdala is highly involved in not only emotional processing, but also cognitive functions. Given the amygdala's vital role in supporting these important cognitive functions, it is unsurprising that it is also diversely connected to subcortical and cortical regions (Sylvester et al. 2020). These numerous connections throughout the brain implicate the amygdala in various invaluable processes, such as attention to salient stimuli, emotional modulation of memory, and cortical sensory plasticity (Phelps and LeDoux 2005), to name a few. Given this broader role of the amygdala in various behaviors, both similar and distinct patterns of androgen related differences in subregion development in adolescent males and females may have important implications for various psychological disorders, including bipolar (Kalmar et al. 2009) and major depressive disorders (Hamilton et al. 2008). Given that most of these disorders show an onset by young adulthood (Kessler et al. 2007), it points to adolescence as a critical period for development. Since structural changes in prefrontal cortex thinning (Bos et al. 2018), as well as amygdala volumes have been linked to psychiatric disorders in adolescence, future research could investigate the importance of considering testosterone, age, and genetic AR sensitivity in further investigating how individual variability in the development of fronto-amygdala circuitry may relate to, and/or act as a brain phenotype, for several mental health disorders.

Even though our work investigates a novel potential phenomenon concerning amygdala subregions, our study has limitations. First, most of our results were considered small effects and became trend-level after correction for multiple comparison; thus, a future study with a larger independent sample size could help validate whether these findings are reproducible. Even if the effects of hormones, age, and AR sensitivity on amygdala development are small, our findings may still have practical significance as many clinically important effects are found to be small in both the fields of psychology and neuroimaging (Meyer et al. 2001, Miller et al. 2016). Second, we found less robust associations with testosterone and no associations with estradiol in females. This could be accounted for by only having one time point for the collection of our estradiol sample. To account for the cyclicity of hormone release for females throughout their cycle, hormone samples and brain scans occurred during the first 10 days of the menstrual cycle when applicable. Therefore, estradiol is inherently at its lowest value, meaning the current study design may have reduced the necessary variance needed to detect the potential associations between hormones levels and amygdala volumes (Dubol et al. 2021). Moreover, recent research in adolescents has found that subcortical regions – such as the amygdala – may be influenced by fluctuating hormone levels (Rehbein et al. 2021), which we did not capture in the current study. In future studies, it would be helpful to have numerous timepoints of collected hormonal data to obtain a more accurate description of individual variability over time. Thirdly, we implemented a standard approach in which we assumed a constant for the albumin concentration for each individual. However, future research should consider measuring individual levels of albumin to reduce any potential effect this assumption may have on the estimation of the bound vs. unbound (bioavailable) quantities of testosterone. As previously published (Shah et al. 2008), we utilized average CAG repeats from both X-alleles to estimate AR sensitivity of females. However, a challenge for this field is understanding the X-chromosome non-random and random inactivation of the AR gene given that it can vary between females and should be investigated in future work (Lappalainen et al. 2008). It should also be noted that the current research study does not account for the potential influence of estrogen receptors, which animal studies suggest may influence masculinization of the brain (Naftolin et al. 1975, Zuloaga et al. 2008) and brain subregional

differences (McCarthy et al. 2017); although, a review of rodent literature demonstrates that assessment of ER sensitivity generally is not fully understood (Wall et al. 2014). In addition, while there are previous structural and functional findings supporting both amygdala associations that are lateral (Canli et al. 2002, Cahill et al. 2004, Neufang et al. 2009, Herting et al. 2014) and bilateral (Whittle et al. 2008, Wierenga et al. 2018), our results support the latter. Though, given our moderate sample size, our bilateral findings should also be replicated in an independent sample to test whether possible unilateral associations may explain significantly more variance and improve model fits in a larger sample. Moreover, future research would also benefit from a longitudinal design, given that a cross-sectional study cannot examine how changes in testosterone levels relate to brain development over time and hopefully increase our power to address these complex modeling questions. Furthermore, cross-sectional vs. longitudinal analyses in even the same study sample have been shown to give different results (Bramen et al. 2012, Herting et al. 2014), highlighting the need to not only study between-subject effects but also within-subject difference over time in multiple samples in order to improve both reproducibility and generalizability of findings (Herting et al. 2018).

5. Conclusion

This study is the first to examine the potential role of bioavailable testosterone levels as well as genetic differences in AR sensitivity in relation to amygdala subregion volumes. In the current adolescent sample, these two androgen derived mechanisms moderated age-related differences in basolateral complex and cortico-medial subnuclei volumes in males, whereas low AR sensitivity was linked to larger basolateral complex volumes across all females regardless of their age or current circulating testosterone levels. The subregion specific patterns in the current study may also emphasize potential heterogeneity of androgen-related sex differences in amygdala development, which may ultimately influence sex-specific patterns of behavior. Given that most of our current findings did not pass multiple comparison corrections, future work is needed to evaluate our numerous findings of small to moderate effects in larger independent cohorts.

Bibliography

1. Ackerman, C. M., L. P. Lowe, H. Lee, F. Chen, E. Hughes, P. Cholod, A. R. Dyer, M. G. Hayes, B. E. Metzger, W. L. Lowe and M. Urbanek (2010). "The role of the polycystic ovary syndrome susceptibility locus D19S884 allele 8 in maternal glycemia and fetal size." *J Clin Endocrinol Metab* **95**(7): 3242-3250.
2. Ahmed, E. I., J. L. Zehr, K. M. Schulz, B. H. Lorenz, L. L. DonCarlos and C. L. Sisk (2008). "Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions." *Nat Neurosci* **11**(9): 995-997.
3. Avants, B. B., N. J. Tustison, G. Song, P. A. Cook, A. Klein and J. C. Gee (2011). "A reproducible evaluation of ANTs similarity metric performance in brain image registration." *Neuroimage* **54**(3): 2033-2044.
4. Avino, T. A., N. Barger, M. V. Vargas, E. L. Carlson, D. G. Amaral, M. D. Bauman and C. M. Schumann (2018). "Neuron numbers increase in the human amygdala from birth to adulthood, but not in autism." *Proc Natl Acad Sci U S A* **115**(14): 3710-3715.
5. Backhausen, L. L., M. M. Herting, J. Buse, V. Roessner, M. N. Smolka and N. C. Vetter (2016). "Quality Control of Structural MRI Images Applied Using FreeSurfer-A Hands-On Workflow to Rate Motion Artifacts." *Front Neurosci* **10**: 558.
6. Bender, R. A., L. Zhou, R. Vierk, N. Brandt, A. Keller, C. E. Gee, M. K. E. Schäfer and G. M. Rune (2017). "Sex-Dependent Regulation of Aromatase-Mediated Synaptic Plasticity in the Basolateral Amygdala." *The Journal of Neuroscience* **37**(6): 1532.
7. Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society. Series B (Methodological)* **57**(1): 289-300.
8. Bos, M. G. N., S. Peters, F. C. van de Kamp, E. A. Crone and C. K. Tamnes (2018). "Emerging depression in adolescence coincides with accelerated frontal cortical thinning." *Journal of child psychology and psychiatry, and allied disciplines* **59**(9): 994-1002.
9. Bramen, J. E., J. A. Hranilovich, R. E. Dahl, J. Chen, C. Rosso, E. E. Forbes, I. D. Dinov, C. M. Worthman and E. R. Sowell (2012). "Sex matters during adolescence: testosterone-related cortical thickness maturation differs between boys and girls." *PLoS One* **7**(3): e33850.
10. Cahill, L., M. Uncapher, L. Kilpatrick, M. T. Alkire and J. Turner (2004). "Sex-related hemispheric lateralization of amygdala function in emotionally influenced memory: an fMRI investigation." *Learn Mem* **11**(3): 261-266.
11. Campbell, C. E., A. F. Mezher, S. P. Eckel, J. M. Tyszka, W. M. Pauli, B. J. Nagel and M. M. Herting (2021). "Restructuring of amygdala subregion apportion across adolescence." *Developmental Cognitive Neuroscience* **48**: 100883.
12. Canli, T., J. E. Desmond, Z. Zhao and J. D. Gabrieli (2002). "Sex differences in the neural basis of emotional memories." *Proc Natl Acad Sci U S A* **99**(16): 10789-10794.
13. Chamberlain, N. L., E. D. Driver and R. L. Miesfeld (1994). "The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function." *Nucleic Acids Res* **22**(15): 3181-3186.
14. Domenech, P. and E. Koechlin (2015). "Executive control and decision-making in the prefrontal cortex." *Current Opinion in Behavioral Sciences* **1**: 101-106.
15. Dormann, C. G., J. Elith, S. Bacher, C. Buchmann, G. Carl, G. Carré, J. García Márquez, B. Gruber, B. Lafourcade, P. Leitão, T. Münkemüller, C. McClean, P. Osborne, B. Reineking, B. Schröder, A. Skidmore, D. Zurell and S. Lautenback (2012). "Collinearity: a review of methods to deal with it and a simulation study evaluating their performance." *Ecography* **35**: 001-020.

16. Dubol, M., C. N. Epperson, J. Sacher, B. Pletzer, B. Derntl, R. Lanzenberger, I. Sundström-Poromaa and E. Comasco (2021). "Neuroimaging the menstrual cycle: A multimodal systematic review." *Frontiers in Neuroendocrinology* **60**: 100878.
17. Elmlinger, M. W., W. Kühnel and M. B. Ranke (2002). "Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults." *Clin Chem Lab Med* **40**(11): 1151-1160.
18. Felten, D. L., M. K. O'Banion and M. S. Maida (2016). 16 - Autonomic-Hypothalamic-Limbic Systems. *Netter's Atlas of Neuroscience (Third Edition)*. D. L. Felten, M. K. O'Banion and M. S. Maida. Philadelphia, Elsevier: 421-461.
19. Field, A. (2013). *Discovering statistics using IBM SPSS Statistics*. London, SAGE.
20. Fowler, C. D., M. E. Freeman and Z. Wang (2003). "Newly proliferated cells in the adult male amygdala are affected by gonadal steroid hormones." *J Neurobiol* **57**(3): 257-269.
21. Giedd, J. N., A. C. Vaituzis, S. D. Hamburger, N. Lange, J. C. Rajapakse, D. Kaysen, Y. C. Vauss and J. L. Rapoport (1996). "Quantitative MRI of the temporal lobe, amygdala, and hippocampus in normal human development: ages 4-18 years." *J Comp Neurol* **366**(2): 223-230.
22. Hamilton, J. P., M. Siemer and I. H. Gotlib (2008). "Amygdala volume in major depressive disorder: a meta-analysis of magnetic resonance imaging studies." *Molecular psychiatry* **13**(11): 993-1000.
23. Herting, M. M., P. Gautam, J. M. Spielberg, E. Kan, R. E. Dahl and E. R. Sowell (2014). "The role of testosterone and estradiol in brain volume changes across adolescence: a longitudinal structural MRI study." *Hum Brain Mapp* **35**(11): 5633-5645.
24. Herting, M. M., C. Johnson, K. L. Mills, N. Vijayakumar, M. Dennison, C. Liu, A. L. Goddings, R. E. Dahl, E. R. Sowell, S. Whittle, N. B. Allen and C. K. Tamnes (2018). "Development of subcortical volumes across adolescence in males and females: A multisample study of longitudinal changes." *Neuroimage* **172**: 194-205.
25. Herting, M. M. and E. R. Sowell (2017). "Puberty and structural brain development in humans." *Front Neuroendocrinol* **44**: 122-137.
26. Hollingshead, A. A. (1975). Four-factor index of social status, Yale University.
27. Johnson, R. T., S. M. Breedlove and C. L. Jordan (2013). "Androgen receptors mediate masculinization of astrocytes in the rat posterodorsal medial amygdala during puberty." *J Comp Neurol* **521**(10): 2298-2309.
28. Juraska, J. M., C. L. Sisk and L. L. DonCarlos (2013). "Sexual differentiation of the adolescent rodent brain: hormonal influences and developmental mechanisms." *Horm Behav* **64**(2): 203-210.
29. Kaczurkin, A. N., A. Raznahan and T. D. Satterthwaite (2019). "Sex differences in the developing brain: insights from multimodal neuroimaging." *Neuropsychopharmacology* **44**(1): 71-85.
30. Kalmar, J. H., F. Wang, L. G. Chepenik, F. Y. Womer, M. M. Jones, B. Pittman, M. P. Shah, A. Martin, R. T. Constable and H. P. Blumberg (2009). "Relation between amygdala structure and function in adolescents with bipolar disorder." *Journal of the American Academy of Child and Adolescent Psychiatry* **48**(6): 636-642.
31. Kessler, R. C., G. P. Amminger, S. Aguilar-Gaxiola, J. Alonso, S. Lee and T. B. Ustün (2007). "Age of onset of mental disorders: a review of recent literature." *Current opinion in psychiatry* **20**(4): 359-364.
32. Khairullah, A., L. C. Klein, S. M. Ingle, M. T. May, C. A. Whetzel, E. J. Susman and T. Paus (2014). "Testosterone trajectories and reference ranges in a large longitudinal sample of male adolescents." *PLoS One* **9**(9): e108838.

33. Lappalainen, S., P. Utriainen, T. Kuulasmaa, R. Voutilainen and J. Jääskeläinen (2008). "Androgen Receptor Gene CAG Repeat Polymorphism and X-Chromosome Inactivation in Children with Premature Adrenarche." *The Journal of Clinical Endocrinology & Metabolism* **93**(4): 1304-1309.
34. Lauretta, R., M. Sansone, A. Sansone, F. Romanelli and M. Appetecchia (2018). "Gender in Endocrine Diseases: Role of Sex Gonadal Hormones." *International journal of endocrinology* **2018**: 4847376-4847376.
35. Lenroot, R. K. and J. N. Giedd (2010). "Sex differences in the adolescent brain." *Brain Cogn* **72**(1): 46-55.
36. Manuck, S. B., A. L. Marsland, J. D. Flory, A. Gorka, R. E. Ferrell and A. R. Hariri (2010). "Salivary testosterone and a trinucleotide (CAG) length polymorphism in the androgen receptor gene predict amygdala reactivity in men." *Psychoneuroendocrinology* **35**(1): 94-104.
37. McCarthy, M., G. De Vries and N. Forger (2017). *Sexual Differentiation of the Brain: A Fresh Look at Mode, Mechanisms, and Meaning*: 3-32.
38. McHenry, J., N. Carrier, E. Hull and M. Kabbaj (2014). "Sex differences in anxiety and depression: role of testosterone." *Frontiers in neuroendocrinology* **35**(1): 42-57.
39. Meyer, G. J., S. E. Finn, L. D. Eyde, G. G. Kay, K. L. Moreland, R. R. Dies, E. J. Eisman, T. W. Kubiszyn and G. M. Reed (2001). "Psychological testing and psychological assessment: A review of evidence and issues." *American Psychologist* **56**(2): 128-165.
40. Miller, K. L., F. Alfaro-Almagro, N. K. Bangerter, D. L. Thomas, E. Yacoub, J. Xu, A. J. Bartsch, S. Jbabdi, S. N. Sotiropoulos, J. L. R. Andersson, L. Griffanti, G. Douaud, T. W. Okell, P. Weale, I. Dragonu, S. Garratt, S. Hudson, R. Collins, M. Jenkinson, P. M. Matthews and S. M. Smith (2016). "Multimodal population brain imaging in the UK Biobank prospective epidemiological study." *Nature Neuroscience* **19**(11): 1523-1536.
41. Naftolin, F., K. J. Ryan, I. J. Davies, Z. Petro and M. Kuhn (1975). "The formation and metabolism of estrogens in brain tissues." *Adv Biosci* **15**: 105-121.
42. Naimi, B., N. Hamm, T. Groen, A. Skidmore and A. Toxopeus (2013). "Where is positional uncertainty a problem for species distribution modeling?" *Ecography* **37**: no-no.
43. Nakagawa, S. and I. C. Cuthill (2007). "Effect size, confidence interval and statistical significance: a practical guide for biologists." *Biol Rev Camb Philos Soc* **82**(4): 591-605.
44. Neufang, S., K. Specht, M. Hausmann, O. Gunturkun, B. Herpertz-Dahlmann, G. R. Fink and K. Konrad (2009). "Sex differences and the impact of steroid hormones on the developing human brain." *Cereb Cortex* **19**(2): 464-473.
45. Paus, T., I. Nawaz-Khan, G. Leonard, M. Perron, G. B. Pike, A. Pitiot, L. Richer, E. Susman, S. Veillette and Z. Pausova (2010). "Sexual dimorphism in the adolescent brain: Role of testosterone and androgen receptor in global and local volumes of grey and white matter." *Horm Behav* **57**(1): 63-75.
46. Perrin, J. S., P. Y. Herve, G. Leonard, M. Perron, G. B. Pike, A. Pitiot, L. Richer, S. Veillette, Z. Pausova and T. Paus (2008). "Growth of white matter in the adolescent brain: role of testosterone and androgen receptor." *J Neurosci* **28**(38): 9519-9524.
47. Phelps, E. A. and J. E. LeDoux (2005). "Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior." *Neuron* **48**(2): 175-187.
48. Preston, A. R. and H. Eichenbaum (2013). "Interplay of hippocampus and prefrontal cortex in memory." *Current biology : CB* **23**(17): R764-R773.
49. Raznahan, A., Y. Lee, R. Stidd, R. Long, D. Greenstein, L. Clasen, A. Addington, N. Gogtay, J. L. Rapoport and J. N. Giedd (2010). "Longitudinally mapping the influence of sex and androgen signaling on the dynamics of human cortical maturation in adolescence." *Proc Natl Acad Sci U S A* **107**(39): 16988-16993.

50. Rehbein, E., J. Hornung, I. Sundström Poromaa and B. Derntl (2021). "Shaping of the Female Human Brain by Sex Hormones: A Review." *Neuroendocrinology* **111**(3): 183-206.
51. Sah, P., E. S. Faber, M. Lopez De Armentia and J. Power (2003). "The amygdaloid complex: anatomy and physiology." *Physiol Rev* **83**(3): 803-834.
52. Shah, N. A., H. J. Antoine, M. Pall, K. D. Taylor, R. Azziz and M. O. Goodarzi (2008). "Association of Androgen Receptor CAG Repeat Polymorphism and Polycystic Ovary Syndrome." *The Journal of Clinical Endocrinology & Metabolism* **93**(5): 1939-1945.
53. Simerly, R. B., C. Chang, M. Muramatsu and L. W. Swanson (1990). "Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study." *J Comp Neurol* **294**(1): 76-95.
54. Sylvester, C. M., Q. Yu, A. B. Srivastava, S. Marek, A. Zheng, D. Alexopoulos, C. D. Smyser, J. S. Shimony, M. Ortega, D. L. Dierker, G. H. Patel, S. M. Nelson, A. W. Gilmore, K. B. McDermott, J. J. Berg, A. T. Drysdale, M. T. Perino, A. Z. Snyder, R. V. Raut, T. O. Laumann, E. M. Gordon, D. M. Barch, C. E. Rogers, D. J. Greene, M. E. Raichle and N. U. F. Dosenbach (2020). "Individual-specific functional connectivity of the amygdala: A substrate for precision psychiatry." *Proceedings of the National Academy of Sciences* **117**(7): 3808.
55. Tyszka, J. M. and W. M. Pauli (2016). "In vivo delineation of subdivisions of the human amygdaloid complex in a high-resolution group template." *Hum Brain Mapp* **37**(11): 3979-3998.
56. Vermeersch, H., G. T'Sjoen, J. M. Kaufman, J. Vincke and M. Van Houtte (2010). "Testosterone, androgen receptor gene CAG repeat length, mood and behaviour in adolescent males." *Eur J Endocrinol* **163**(2): 319-328.
57. Vermeulen, A., L. Verdonck and J. M. Kaufman (1999). "A critical evaluation of simple methods for the estimation of free testosterone in serum." *J Clin Endocrinol Metab* **84**(10): 3666-3672.
58. Vigil, P., J. P. Del Rio, B. Carrera, F. C. Aranguiz, H. Rioseco and M. E. Cortes (2016). "Influence of sex steroid hormones on the adolescent brain and behavior: An update." *Linacre Q* **83**(3): 308-329.
59. Volman, I., I. Toni, L. Verhagen and K. Roelofs (2011). "Endogenous testosterone modulates prefrontal-amygdala connectivity during social emotional behavior." *Cerebral cortex (New York, N.Y. : 1991)* **21**(10): 2282-2290.
60. Wall, E. H., S. C. Hewitt, L. K. Case, C.-Y. Lin, K. S. Korach and C. Teuscher (2014). "The role of genetics in estrogen responses: a critical piece of an intricate puzzle." *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **28**(12): 5042-5054.
61. Whittle, S., M. B. Yap, M. Yucel, A. Fornito, J. G. Simmons, A. Barrett, L. Sheeber and N. B. Allen (2008). "Prefrontal and amygdala volumes are related to adolescents' affective behaviors during parent-adolescent interactions." *Proc Natl Acad Sci U S A* **105**(9): 3652-3657.
62. Wierenga, L. M., M. G. N. Bos, E. Schreuders, F. Vd Kamp, J. S. Peper, C. K. Tamnes and E. A. Crone (2018). "Unraveling age, puberty and testosterone effects on subcortical brain development across adolescence." *Psychoneuroendocrinology* **91**: 105-114.
63. Zuloaga, D. G., D. A. Puts, C. L. Jordan and S. M. Breedlove (2008). "The role of androgen receptors in the masculinization of brain and behavior: what we've learned from the testicular feminization mutation." *Horm Behav* **53**(5): 613-626.

Figure Legends

Figure A.1: 3D and graphical representation of results in females. 1) Coronal 3D representation of the androgen receptor sensitivity results. 2) Graphical representation of subregions with small AR effects from panel (1); for visualization purposes, each individual is plotted (black dots) and lines represent predicted values of volume in relationship to AR repeat length (inversely related to sensitivity), with mean values of each covariate of non-interest (i.e., SES and ICV) held constant; dashed lines represent the 95% Confidence Intervals of the model prediction line; ω^2 represents the effect size of the highest order interaction term.

Abbreviations: AR, androgen receptor; LA, lateral nucleus; BLDI, basolateral dorsal and intermediate subdivision; BLVPL, basolateral ventral and paralamina subdivision; BM, basomedial nucleus.

* FDR p-value ≤ 0.05 (significant)

Figure A.2: 3D and graphical representation of results in males. 1) Coronal 3D representation of androgen receptor sensitivity and free testosterone interaction results. 2) Graphical representation of small and medium effects from panel plotted in the low AR sensitivity group and the high (1); FT concentrations plotted were based on FT intra-quartile range values (Q1 = 0.12; Q3 = 0.35); for visualization purposes, each individual is plotted (black dots) and lines represent predicted values of volume at a given FT level, with mean values of each covariate of non-interest (i.e., SES and ICV) held constant; dashed lines represent the 95% Confidence Intervals of the model prediction line; ω^2 represents the effect size of the highest order interaction term.

Abbreviations: FT, free testosterone; AR, androgen receptor; BLVPL, basolateral ventral and paralaminar subdivision; CMN, cortical and medial nuclei.

* FDR p-value ≤ 0.05 (significant)

Highlights:

- Testosterone and androgen receptor genotype relate to basolateral amygdala volumes
- Testosterone relates to less age related decrease in volumes in males
- Main effect of androgen receptor genotype on volumes more prevalent in females
- Higher androgen receptor sensitivity associated with smaller volumes