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Testosterone effects on functional amygdala lateralization: A study in adolescent transgender boys and cisgender boys and girls

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ABSTRACT

The influence of testosterone on the development of human brain lateralization has been subject of debate for a long time, partly because studies investigating this are necessarily mostly correlational. In the present study we used a quasi-experimental approach by assessing functional brain lateralization in trans boys (female sex assigned at birth, diagnosed with Gender Dysphoria, n = 21) before and after testosterone treatment, and compared these results to the functional lateralization of age-matched control groups of cisgender boys (n = 20) and girls (n = 21) around 16 years of age. The lateralization index of the amygdala was determined with functional magnetic resonance imaging (fMRI) during an emotional face matching task with angry and fearful faces, as the literature indicates that boys show more activation in the right amygdala than girls during the perception of emotional faces. As expected, the lateralization index in trans boys shifted towards the right amygdala after testosterone treatment, and the cumulative dose of testosterone treatment correlated significantly with amygdala lateralization after treatment. However, we did not find any significant group differences in lateralization and endogenous testosterone concentrations predicted rightward amygdala lateralization only in the cis boys, but not in cis girls or trans boys. These inconsistencies may be due to sex differences in sensitivity to testosterone or its metabolites, which would be a worthwhile course for future studies.

1. Introduction

The two hemispheres of the brain differ in structure and function, with small but robust differences between the sexes in functional lateralization (meta analysis Hirnstein et al., 2018). Based on this sex difference, the development of brain lateralization has long been thought to be under the influence of testosterone (for a review see Pfannkuche et al., 2009). Two main theories provide a background for this idea, the callosal theory and the sexual differentiation theory. The callosal theory (Witelson and Nowakowski, 1991) assumes that prenatal testosterone affects axonal pruning in the corpus callosum, thereby reducing the number of connections between the hemispheres, leading to stronger lateralization. The sexual differentiation theory (Hines and Shipley, 1984) explains differences in lateralized functions to be related intrinsically to sexual differentiation, both being affected by prenatal sex hormones. The oldest hypothesis, by Geschwind and Galaburda (1985) explained the sex difference in lateralization as the consequence of testosterone differentially influencing the rate of development of both hemispheres, but little evidence has been found to support it (Bryden et al., 1994; Pfannkuche et al., 2009).

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However, studies investigating the influence of testosterone on lateralization in humans are scarce and mostly correlational in nature (Beking et al., 2017). In the current study we use a quasi-experimental approach, by investigating the effect of testosterone treatment on functional lateralization in trans boys (female sex assigned at birth, diagnosed with Gender Dysphoria, American Psychiatric Association, 2013).

The amygdala is part of the limbic system and involved in emotion and memory. The amygdala is one of the most studied brain areas for which structural asymmetries have been reported. From childhood to young adulthood, lateralized sex differences in structure have been found, namely, the right amygdala volume was larger than the left amygdala in boys, whereas there was no difference between the left and right amygdala volume in girls (Uematsu et al., 2012). In adulthood, however, there is no sex difference in structural asymmetry according to a recent large meta-analysis including 15.847 participants (Guadalupe et al., 2016). In both sexes, the volume of the right amygdala is larger than the left amygdala. Some primary studies indicate that connections to other brain areas are more widespread from the right amygdala in men, and from the left amygdala in women (Kilpatrick et al., 2006; Savic and Lindström, 2008), and that neuron size is larger in the right amygdala in men and in the left amygdala in women (Antyukhov, 2016).

Both amygdalae are involved in emotion processing, but which side is more dominant seems to depend on many factors, such as valence and sex of the stimuli, and whether conscious or automatic processing is involved (Sergerie et al., 2008). Nevertheless, many studies demonstrated a quite consistent sex difference in lateralized activity in the amygdalae. A meta-analysis showed that males are overall stronger lateralized, that males showed more activation peaks in the right amygdala and females in the left amygdala, and that both amygdalae are part of an active network during emotion processing (Wager et al., 2003). In men, this network included the amygdala in the right hemisphere, and in women the amygdala in the left hemisphere (Cahill et al., 2004; Canli et al., 2002). Schneider et al. (2011) and Killgore and Yurgelun-Todd (2004) found a stronger right than left amygdala activation in males, but no effect in females. In contrast, a meta-analysis including 148 studies found no sex effect in lateralization of the amygdalae, but did find a larger effect size of overall amygdalae activation in men than in women (Sergerie et al., 2008).

One of the key functions of the amygdala is the processing of facial emotional expressions (Fusar-Poli et al., 2009). A large study specifically investigating the perception of angry faces in 470 adolescent boys and girls, found a stronger right than left amygdala activation in boys, but no asymmetrical activation in girls (Schneider et al., 2011). This was consistent with a study investigating perception of fearful faces (Killgore and Yurgelun-Todd, 2004). In the present study we used a face matching task with angry and fearful faces (Hariri et al., 2000).

Puberty is a particularly interesting period to study effects of testosterone, as it has been suggested that this is a second sensitive period in which sex hormones affect the sexual differentiation of the brain (Peper et al., 2011). A previous longitudinal study of our research group in adolescents reported effects of prenatal and pubertal testosterone on lateralized brain activity, using functional Transcranial Doppler ultrasonography (Beking et al., 2018). A more direct test of the effect of testosterone on functional lateralization is the administration of testosterone. In the present study we investigated the effect of testosterone treatment in adolescent trans boys, who experience a severe incongruence between their sex assigned at birth and their gender identity. From around 16 years of age, after careful diagnostic evaluation and consultation, they may choose to start hormone treatment. A masculinizing puberty is then induced by administering testosterone (Kreukels et al., 2011). This is a unique group of participants to investigate the effect of testosterone treatment on emotion processing, and, more specifically, in terms of lateralization of associated amygdala activation.

Only one study investigated the effect of steroid hormone treatment on lateralization of brain activation in persons diagnosed with Gender Dysphoria (Sommer et al., 2008). The authors investigated the effect of estrogen treatment in 8 transwomen (male assigned at birth, female gender identity) and of testosterone treatment in 6 transmen (female assigned at birth, male gender identity) on lateralization of mental rotation and language as measured with functional magnetic resonance imaging (fMRI). Functional lateralization of both tasks was compared before treatment and after three months of cross-sex hormone treatment (parenteral testosterone esters (Sustanon) 250 mg/ml every 14 days). No effects of cross-sex hormone treatment on lateralization were found (Sommer et al., 2008). In the present study, we investigated the effect of 10 months of testosterone treatment during puberty (instead of adulthood) on functional lateralization of the amygdala during emotional face processing – arguably a more basic function than visuospatial cognition and language. Moreover, the sample size of our study is considerably larger and, in contrast to the Sommer et al. (2008) study, outcomes are compared to control groups of both cisgender (i.e., sex assigned at birth and experienced gender are congruent) boys and girls.

To our knowledge, there is no literature investigating the effect of long-term testosterone administration on amygdala activation. Short-term single testosterone administration studies in adult women found an increase in bilateral amygdala reactivity during an emotional face matching task with angry and fearful faces. Androgen levels were lower in middle-aged than young women, which was associated with decreased amygdala reactivity. The single nasal dose of testosterone increased amygdala reactivity to a level comparable to that of young women (van Wingen et al., 2009). Lateralized effects of sublingual testosterone administration have been found in young women on specifically the right amygdala while watching angry faces (versus happy faces) (Hermans et al., 2008), and watching movies of faces that changed their expression from neutral to emotional (either happy or fearful) (Bos et al., 2013).

Previous studies from our group (including partly the same participants as in the current study) found that during cognitive tasks for which cisgender sex differences have been observed, adolescent trans boys showed brain activation patterns that were “in-between” that of cis male and cis female control groups, i.e., neither sex-typical nor sex-atypical (Burke et al., 2016; Soleman et al., 2013; Staphorsius et al., 2015). However, none of these studies analyzed lateralization effects as a function of sex and gender identity, and studies of the effects of cross-sex hormone treatment on brain and cognition are very limited, in particular with regard to adolescent brain development. Another study investigated behavioural lateralization (measured with dichotic listening) of 44 adult transwomen and 34 adult transmen before hormone treatment and found that the participants diagnosed with Gender Dysphoria were on average less lateralized than the control groups (Cohen-Kettenis et al., 1998). On the other hand, Herman et al. (1993) found that biological sex and gender identity were unrelated to the pattern of hemispheric lateralization. The present study will explore this relation for functional brain lateralization (fMRI) rather than behavioural lateralization, as well as the effect of long-term testosterone treatment.

So far, studies that investigated the effects of sex hormones on amygdala activation did not take the relative activation between the left and right amygdala – i.e., a lateralization index (LI) - into account. This index is, however, a widely used standard to measure the degree and direction of lateralization. In the present study, we specifically aimed to investigate the effect of testosterone treatment in trans boys on functional lateralization of the amygdala and compare their LI within the same subjects before treatment and with the LI of cisgender male and female control groups, as well as the relationship between LI and actual testosterone concentrations as measured in saliva.

We hypothesize that 1a) There is a sex difference in functional lateralization of amygdala activations, with cis boys showing a stronger

rightward lateralization than cis girls; 1b) Transboys have a weaker amygdala lateralization than cis boys and girls before testosterone treatment (based on Cohen-Kettenis et al., 1998); 2) There is a correlation between testosterone levels and functional rightward lateralization of amygdala activation in the control groups, especially in the cis boys; 3) Testosterone treatment in trans boys will shift lateralization of amygdala activation towards the right hemisphere.

Trans boys – after puberty suppression – performed an emotional face matching task just before and again after on average ten months of treatment with testosterone. We collected similar data in cisgender boys and girls and first tested for whole-brain group differences in brain activation during emotion processing within and between sessions. Then, focusing on the amygdala as our region of interest, the LI was calculated from individual amygdalae responses during emotional face matching trials.

2. Method

2.1. Participants

Trans boys were recruited via the Center of Expertise on Gender Dysphoria, VU University Medical Center in Amsterdam, the Netherlands. Age-matched cisgender boys and girls were recruited via secondary schools and by inviting friends of the trans boys. All trans boys identified as male and met the diagnostic criteria for Gender Dysphoria (DSM-5, American Psychiatric Association, 2013; at that time DSM-IV-TR criteria were still employed), all cisgender adolescents had a gender identity in line with their birth assigned sex. Exclusion criteria for participation in the study were continuous psychotropic medication use, and any form of psychiatric or neurologic disorder. At the first session, 21 trans boys (M age = 16.1 years, SD = 0.7), 20 cis boys (M age = 15.9, SD = 0.6) and 21 cis girls (M age = 16.4, SD = 1.0) participated. One year later, all trans boys participated again, and 3 cis boys and 1 cis girl dropped out. All participants gave their informed consent and ethical clearance was given.

At the first session, 1 cis boy and 1 cis girl were excluded because the amygdala did not show activation during the task and a lateralization index could not be determined. At the second session, 1 cis girl was excluded due to technical problems with the fMRI acquisitions. The final sample used for analyses consisted of 21 trans boys, 19 cis boys and 20 cis girls for session 1, and of 21 trans boys, 17 cis boys and 19 cis girls for session 2.

Participants completed the Dutch translation of the Edinburgh Handedness Inventory (van Strien, 2002) at session 1.

2.2. Hormone treatment

Up to session 1, the trans boys had received 3.75 mg Triptorelin (Decapeptyl-CR®) subcutaneously or intramuscularly every 4 weeks to suppress production of gonadal hormones, and thereby puberty (mean duration = 1.6 years, SD = 1.0).

After session 1 and up to session 2, trans boys received testosterone treatment (M = 9.8 months, SD = 2.9, range 5.6–14.8 months): 14 trans boys received an ester-testosterone mixture every 2 weeks (Sustanon® 250 mg/ml), and 7 trans boys received testosterone undecanoate every 12 weeks (Nebido® 250 mg/ml). Following the guidelines by Hembree et al. (2009), the dosage depended on the participants' age: 25 mg/m^2 body surface area until age 16.5 years, and 75 mg/m^2 from age 16.5 onwards. This gradually increasing dose schedule of cross-sex hormone steroids was employed in order to mimic induction of puberty as much as possible. Cisgender boys and girls received no treatment.

2.3. Hormone assay

At the day of testing, participants were asked to collect 1 ml of saliva in a polypropylene tube directly after waking up, and before eating or tooth brushing. Testosterone levels in saliva were determined with isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC–MS/MS), which has been validated to be a sensitive (lower limit of quantification was approximately 1 fmol (0.3 pg)), specific (only 0.4% interference for a different steroid androstenedione), and accurate (recovery rate of 93 ± 7% (mean ± SD)) method for measuring salivary testosterone levels, see for further details (Bui et al., 2013). Testosterone levels from trans boys were not determined at session 1, because they were under pubertal suppression, that has been proven to result in testosterone levels under detection levels (Soleman et al., 2016).

2.4. Face matching task

Participants performed an fMRI face-matching task (Hariri et al., 2000) that has been shown to robustly engage the amygdala. We chose to investigate fearful and angry faces, as these emotions elicit strong amygdala activation, on which effects of testosterone have been demonstrated (Bos et al., 2012; Derntl et al., 2009; Fusar-Poli et al., 2009). Stimuli were derived from the NimStim set of Facial Expressions (Tottenham et al., 2009). In this task, an angry or fearful target face was presented above 2 horizontally placed reference faces, of which one was fearful and one angry (see Fig. 1A). Participants had to indicate with a left or right button press which of the reference faces showed the same emotion as the target face. All three simultaneously presented faces per trial were from different persons of the same sex and were counterbalanced for ethnicity. Across trials, the faces were counterbalanced for sex and emotion. For analyses, data were pooled across fearful and angry trials. In the control condition participants had to match simple circular shapes, which were displayed in a similar manner as the face stimuli. Participants had to indicate with a left or right button press which of the reference shapes looked the same as the target shape (see Fig. 1B). The task consisted of 4 face matching blocks alternated with 5 control blocks, and there were no breaks in between the blocks. Each block started with an instruction trial announcing the following condition and included six trials with randomly varying inter-trial intervals (jitter). Inter-trial intervals varied between 2, 3, 4, 5, and 6 s for the emotional face trials, and were fixed (2 s) for shape trials. Presentation side (left or right, at the bottom of the screen) of the matching stimulus was counterbalanced across trials. A fixation cross was shown for 1 s after each trial. Each block consisted of 6 trials, the total task duration was about 5 min as timing of the stimuli was self-paced, with maximum response duration of 4 s per trial. Several practice trials were performed outside the scanner to ensure participants comprehended the task.

2.5. Imaging protocol

Scans for session 1 were performed on a 3.0 T GE Signa HDxt scanner (General Electric, Milwaukee, Wisconsin, USA). A gradient-echo echo-planar imaging sequence was used for functional imaging. The parameters included a 19.2 cm^2 field of view, TR of 1950 ms, TE of 25 ms, an 80° flip angle, isotropic voxels of 3 mm, and 36 slices. Before each imaging session a local high-order shimming technique was used to reduce susceptibility artifacts. For co-registration with the functional images a T1-weighted scan was obtained (3D FSPGR sequence, 25cm2 field of view, TR of 7.8 ms, TE of 3.0 ms; slice thickness of 1 mm, and 176 slices). For further description of the procedure see Burke et al. (2016).

During the course of the project, a scanner upgrade (hardware and software) took place (GE scanner, type MR750). Although all settings of the scanning protocol remained unchanged, we counterbalanced session 2 scans over groups in order to account for possible effects of the upgrade (all session 1 scans were performed before the upgrade). The lateralization index did not differ across participants at session 2 before and after the upgrade ($F(1,54) = 3.10$, $p = .084$).

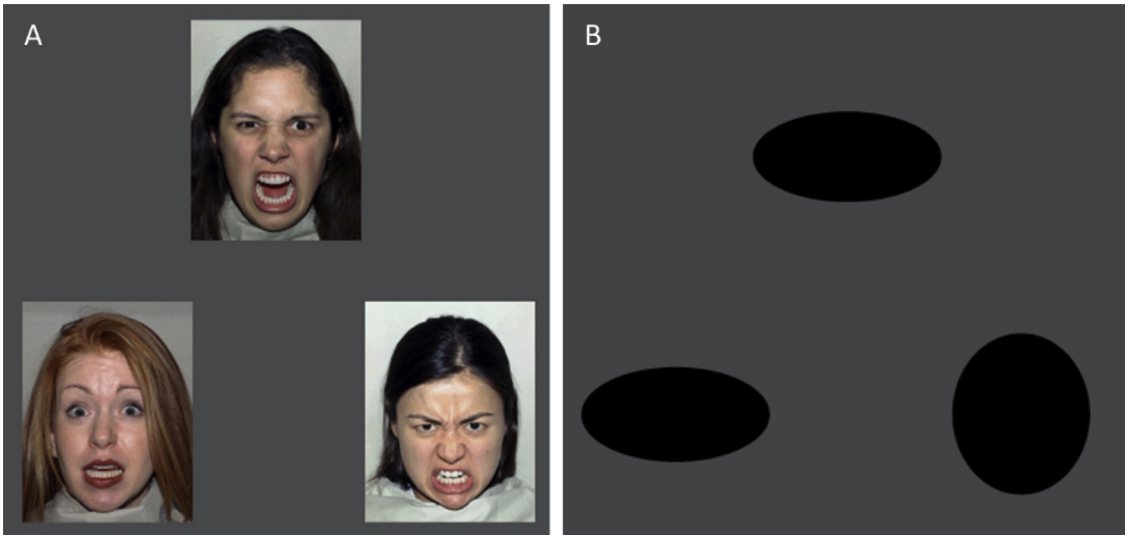

Fig. 1. Emotional face matching task.
Example stimuli of the fMRI emotional face matching task. In the face-matching condition (A), participants indicated by button press with the left or right index finger, which of the two facial cues presented at the bottom of the screen matched the emotion of the target face centered at the top of the screen. In the control condition (B), participants matched simple geometric shapes. All face stimuli were derived from the NimStim set of Facial Expressions (Tottenham et al., 2009).
Note: Size of the full picture presented (full screen) was 842 × 595px, grey color background; Shape stimuli had the following sizes: Ellipse-shaped stimuli 69 × 135px, Roundish stimuli 99 × 115px, Face stimuli: 123 × 160px, Stimulus presented at the top, centered at 415 × 243px, Left-sided stimulus centered at 292 × 419px, Right-sided stimulus centered at 540 × 420px.

2.6. fMRI analyses

FMRI data pre-processing, first-level and group-level analyses were performed with SPM8 software (Statistical Parametric Mapping; Wellcome Department of Imaging Neuroscience, Institute of Neurology at the University College London, UK) implemented in Matlab R2012b (Math Works Inc., Natick, MA, USA).

First, a group template was created for anatomical images acquired during the first and second sessions combined using Diffeomorphic Alignment Registration Exponentiated Lie Algebra (DARTEL) for optimal spatial normalization. Second, standard preprocessing was performed per session, which comprised the following steps: slice time correction, realignment to the functional mean image, co-registration with the individual anatomical image, normalization to the DARTEL template, and smoothing with an 8 mm FWHM kernel size. First-level contrast images were built by subtracting control trials (shapes) from emotion trials (faces). Individual head jerks of more than 1 mm together with the six motion parameters were included as nuisance variables in every first-level design matrix (Lemieux et al., 2007) to account for the effects of excessive head motion. Second-level random effects analyses were performed including all individual contrast images (faces > shapes) and using a flexible factorial design, modeling within-group effects of the factor session, and group by session interaction effects. Thereby, we investigated the effects of testosterone treatment (session 2 versus session 1), while controlling for possible developmental and/or learning effects. Thus, adding both control groups to the design controlled for possible within-subject effects other than the testosterone treatment. We used one-way ANOVA to test for group differences separately per session. Whole brain effects were considered significant at a *p*-value of .05 family-wise error corrected ($pFWE = .05$).

Next, we focused our analyses on the amygdala as our region of interest. Analyses were performed using MATLAB version 2011b and SPM12. We used the LI-Tool (Wilke and Lidzba, 2007) to calculate laterality indices for faces – shapes contrasts for all participants. The amygdala was specified with the WFU Pickatlas version 2.4. The size of the mask of the left and right amygdala was symmetrical. The laterality index (LI) was calculated with the following (default) settings: voxel values were used, thresholding was based on the bootstrapping technique, and a standard exclusion mask with a midline margin of 5 mm was used. The fMRI LI-Tool calculates the LI as follows: LI = (actL-actR)/(actL + actR) with actR and actL being the activation levels in BOLD (blood-oxygen-level-dependent) response of individuals' left and right amygdala mask respectively. We selected the weighted mean lateralization index scores for further analysis.

2.7. Statistical analyses

The statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), version 25. Testosterone concentrations were not normally distributed, so in order to determine the effect of hormone treatment on circulating testosterone concentrations the difference in testosterone concentrations between groups were tested with Mann-Whitney U tests per session, and the change in testosterone concentrations between sessions with a Wilcoxon signed ranks test per group. Based on the literature (Soleman et al., 2016), the testosterone concentration at session 1 was set at the detection limit of 10 nmol/L for trans boys.

Handedness was not normally distributed and differences between the groups were tested with a Kolmogorov-Smirnoff test and Mann-Whitney U tests. As an exploratory analysis, we checked if handedness as a covariate had any effect on the analyses presented in this article, and this was not the case: all outcomes remained qualitatively the same, and handedness as covariate had no significant effect on lateralization. Therefore, we report the models without handedness as covariate.

Lateralization was normally distributed. The difference in lateralization between groups was first tested separately per session with one-way ANOVAs. Then, to test if trans boys – being our quasi-experimental group – differed from cisgender boys and girls in the *change* in lateralization before and after treatment, we performed a repeated measures ANOVA with Session (2 levels) as within-subjects factor and Group (3 levels) as between-subjects factor.

In order to examine the potential relationship between endogenous testosterone concentrations and lateralization indices in the cisgender control groups, we performed a regression analysis with testosterone levels as predictor for LI, separately per group and session. Next, we tested the effect of exogenous testosterone concentrations on

lateralization with a similar regression analysis in trans boys at session 2. In addition, we tested the effect of exogenous testosterone concentrations on the change in lateralization from before to after treatment (difference scores in LI session 2 – session 1) with another regression analysis in the trans boys. Moreover, the cumulative dose of administered testosterone might represent testosterone treatment better than the level measured at session 2. The injected dose per m^2 body surface changes after age 16.5 years, therefore the total dose of testosterone treatment is defined as: (16.5 – age start testosterone treatment)*25 mg/m^2 + (age at session 2–16.5)*75 mg/m^2. The effect of the cumulative dose of testosterone treatment on the change in lateralization from session 1 to 2 was tested with a GLM. The model including only the cumulative dose of testosterone as a predictor fitted better (lower AIC) than a model including both testosterone level at session 2 and the cumulative dose of testosterone, therefore only the first model is presented.

3. Results

Before presenting the results of the data analyses related to our hypotheses on functional amygdala lateralization, we present group differences in testosterone concentrations, explore group differences in the distribution of hand preference, and present findings of the whole-brain activation contrasts of the emotional face matching task.

3.1. Testosterone concentrations

Fig. 2 presents testosterone levels per group per session, confirming sex differences and the effect of testosterone treatment. At both sessions, cis boys had higher testosterone levels than cis girls (session 1: $U = 6.00$, $p < .001$, session 2: $U = 0.00$, $p < .001$). For the cisgender boys, testosterone levels were similar at session 1 and 2 ($Z = -.54$, $p = .586$), and tended to be lower at session 2 in cisgender girls ($Z = -1.78$, $p = .076$). Assuming that testosterone concentrations of trans boys were below detection limit in session 1, testosterone treatment strongly increased testosterone levels at session 2 ($Z = -4.02$, $p < .001$). Their testosterone concentrations were now higher than in cis girls ($U = 0.00$, $p < .001$), and comparable to the levels of cis boys ($U = 142.00$, $p = .284$).

3.2. Handedness

According to the EHI, only 1 participant was left-handed (EHI ≤ -8, trans boy), 18 participants ambidexter (-8 < EHI < 8, 9 trans boys, 5 cis boys, 4 cis girls), and 43 participants right-handed (EHI ≥ 8, 11 trans boys, 15 cis boys, 17 cis girls). Handedness per group: cis boys (M = 8.59, SD = 2.07, range [4–10]), cis girls (M = 8.56, SD = 2.40, range [3–10]), trans boys (M = 5.67, SD = 5.90, range [-9 to 10]). The distribution of handedness did not differ significantly between the groups (all Kolmogorov-Smirnoff $Z < 0.93$, $p > .358$). Trans boys tended to be weaker lateralized for handedness than cis boys (Mann-Whitney U $Z = -1.68$, $p = .094$) and did not differ from cis girls ($Z = -1.50$, $p = .135$). Cisgender boys and girls did not differ in handedness ($Z = -0.37$, $p = .710$).

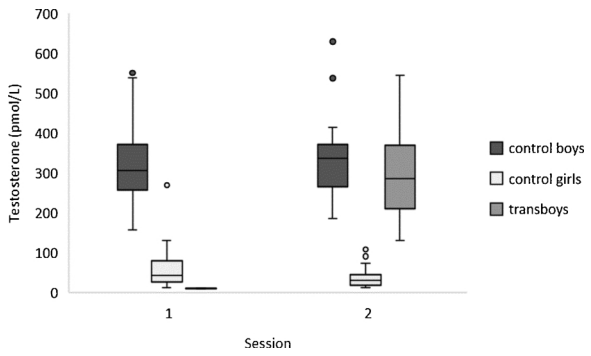

Fig. 2. Boxplot of testosterone levels per group per session. Error bars represent the 95% confidence intervals. Dots represent the outliers > 1.5x interquartile range.

3.3. fMRI task main effect

In both sessions, during emotional face processing, robust task-related bilateral activations were found across groups, recruiting occipito-temporal and frontal regions, including the amygdala, and middle and inferior frontal areas (see Fig. 3 and Table 1).

3.3.1. Whole-brain results

For the contrast *faces - shapes*, at a whole-brain threshold of $p < .05$, *FWE*-corrected, we found significant main effects of session in both directions: across groups, there were significantly stronger activations in the right cerebellum at session 1 compared with session 2 ($t = 10.6$, x y z = 33 -45 -30, 80 voxels); and significantly stronger activation during session 2 compared with session 1 in the bilateral fusiform gyrus (left: $t = 14.2$, x y z = -33 -45 -18, 363 voxels; right: $t = 12.5$, x y z = 36 -66 -9, 404 voxels) and the left amygdala ($t = 8.2$, x y z = -18 -9 -12, 34 voxels). Separate within-group comparisons confirmed the decrease in cerebellar and increase in fusiform activations in each group over time. In addition, in the trans boys, but not in any of the cisgender groups, a between-sessions increase in left amygdala activation passed the threshold for significance ($t = 5.3$, $p_{FWE} = .024$, x y z = -18 -9 -12, 2 voxels). Thus, only in the trans boys we observed relatively stronger amygdala reactivity after 10 months of testosterone treatment, compared to before the start of treatment. We found no significant group by session interaction effects. Also, we found no significant group differences in emotional face processing on the whole-brain level, neither at session 1, nor at session 2.

3.4. Effects of group and session on functional amygdala lateralization

Hypothesis 1a. "There is a sex difference in functional lateralization of amygdala activations, with cis boys showing a stronger rightward lateralization than control girls".

Hypothesis 1b. "Trans boys have a weaker amygdala lateralization than cisgender boys and girls before testosterone treatment".

The average lateralization index per group per session is depicted in Fig. 4. At session 1, a one-way ANOVA comparing amygdala lateralization of the three groups, was not significant ($F(2, 57) = 2.37$, $p = .102$). However, given our clear a priori defined hypotheses, we performed further exploratory two-group comparisons. Contrary to expectation, the difference in lateralization between cisgender boys and girls did not reach significance ($t(57) = 0.72$, $p = .943$. With regard to hypothesis 1b, we found that, according to expectation, trans boys tended to be less lateralized in amygdala activation than cis boys ($t(57) = 1.82$, $p = .073$), and cis girls ($t(57) = 1.92$, $p = .060$) at session 1. Again, not in support of our hypotheses, at session 2, the lateralization index did not differ between any of the groups ($F(2,54) = 0.19$, $p = .825$).

We tested if the change in lateralization from session 1 to 2 was different between trans boys and control groups. A repeated measures ANOVA revealed no significant main effects of Session ($F(1, 52) = 1.01$, $p = .320$) or Group ($F(2, 52) = 1.07$, $p = .484$), but the more important Session by Group interaction, showed an interesting trend ($F(52, 2) = 2.5$, $p = .092$). Because of this trend and the small sample sizes, exploratory follow-up comparisons of individual LI difference scores (LI session 2 – LI session 1) were performed in order to investigate more specific group differences in the change of lateralization. Planned contrasts revealed that trans boys tended to have larger LI difference scores than cis boys ($t(52) = 1.84$, $p = .071$), and cis girls (t

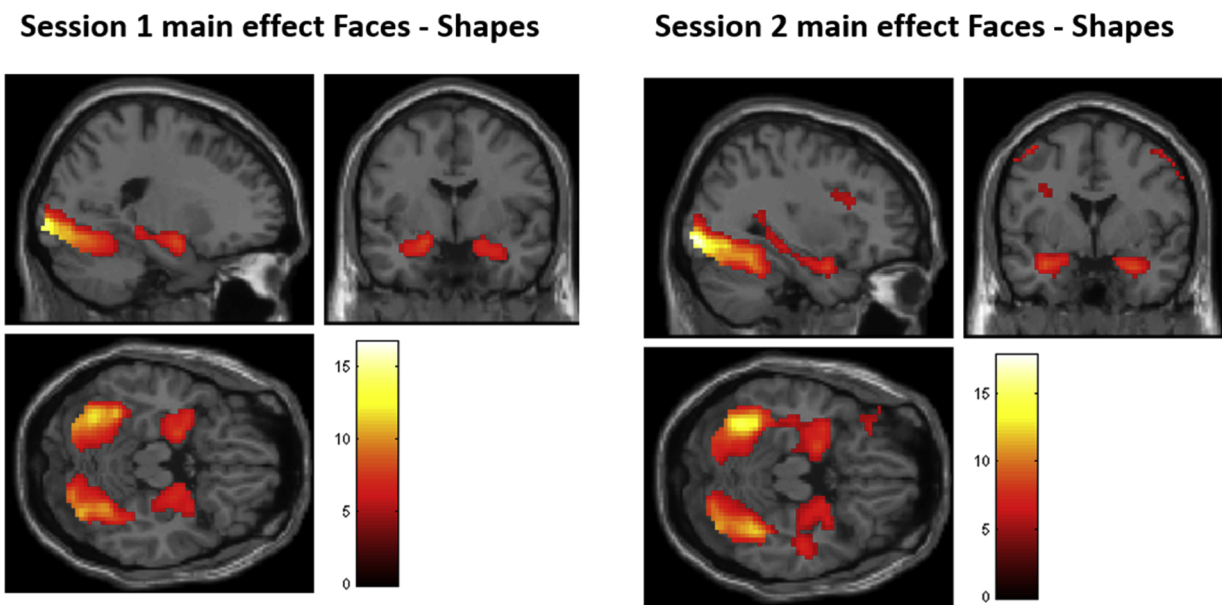

Fig. 3. Brain activations for the main effect of the emotional face matching task, across groups and separately per session showing robust bilateral amygdala engagement. Images were thresholded at FWE-corrected p < .05; the color bars indicate t-value [FWE = family-wise error].

(52) = 1.96, p = .056). As can be seen in Fig. 4, trans boys had a relatively more leftward lateralization in amygdala activation at session 1, and a relatively more rightward lateralization at session 2.

3.5. The relation between endogenous testosterone and lateralization in the control groups

Hypothesis 2. "There is a correlation between testosterone levels and functional rightward lateralization of amygdala activation in the control groups, especially in the cis boys".

For cis boys, testosterone significantly predicted lateralization at session 1 (F(1,17) = 7.77, R^2 = .314, p = .013), but not at session 2 (F(1,15) = 1.78, R^2 = .106, p = .202). For cis girls, testosterone concentrations did not predict lateralization at session 1 (F(1,17) = 1.88, R^2 = .100, p = .188), nor at session 2 (F(1,17) = 1.23, R^2 = .068, p = .282) (Fig. 5).

3.6. The effect of exogenous testosterone treatment on lateralization of amygdala activation in trans boys

Hypothesis 3. "Testosterone treatment in trans boys will shift lateralization of amygdala activation towards the right hemisphere".

The relation between testosterone levels in trans boys at session 2 (which also reflects the *change* in testosterone as testosterone was suppressed at session 1) and the *change* in lateralization from session 1 to 2 in trans boys (LI differences scores) was not significant (F(1,19) = 0.02, R^2 = .001, p = .894).

In addition, the relation between testosterone levels at session 2 and lateralization at session 2 was not significant (F(1,19) = 1.99, R^2 = 0.095, p = .175).

Interestingly, the cumulative dose of testosterone treatment tended to be associated with LI difference scores (session 2 – 1) in trans boys (F(1,19) = 3.39, R^2 = .151, p = .081). Moreover, the cumulative dose of testosterone treatment was significantly related with amygdala lateralization at session 2 (F(1,19) = 5.82, R^2 = .234, p = .026), see Fig. 6. However, this p-value would not survive correction for multiple comparisons: p = 0.05/4 = .013.

4. Discussion

Lateralization is a basic organization principle of the brain throughout the entire animal kingdom and certainly in humans, but its development is elusive. For decades, testosterone has been thought to influence brain lateralization, but this is difficult to investigate in humans. Our group of transgender boys, receiving testosterone as part of their gender-affirming hormone treatment, offered the opportunity to investigate the influence of testosterone administration in humans. The aim of this study was to investigate the effect of testosterone on lateralization of amygdala activation during emotional face perception. To this end we used a quasi-experimental approach by assessing amygdala lateralization in trans boys before and after testosterone treatment, and compared it to control groups of cisgender boys and girls.

First, we showed that our task did activate the relevant brain regions across groups, including the amygdalae. Overall, participants showed stronger task-related brain activations during emotional face matching at the second compared with the first session. More specifically, we found a significant decrease of right cerebellar activation across groups, and an overall increase in activation in the bilateral fusiform gyrus. Functional involvement of the cerebellum in emotional (face) processing has been reported previously (Marusak et al., 2013; Reiman et al., 1997; Turner et al., 2007). In addition, Petersen et al. (1998) suggested that right cerebellar activation was associated with initial unskilled task performance and novel task demands. Therefore, cerebellar involvement may decrease with task experience, and could therefore reflect practice effects.

An opposite pattern of change has previously been reported for face-processing brain regions: more experience with face processing over the course of development was associated with increased activations in face-processing neural regions (Scherf et al., 2013), which is in line with our results of increased bilateral fusiform gyrus activations in session 2 compared with session 1. Moreover, it is assumed that the fusiform gyrus is particularly recruited when the perceiver has learned to discriminate individual facial identities, which thus again reflects effects of experience (Tarr and Gauthier, 2000).

Interestingly, on whole-brain level, and although the cluster that survived the threshold for significance was small, we observed significantly stronger amygdala activation in session 2 only in the trans boys, thus after testosterone treatment and compared to session 1, when they had received hormone suppressants. This may indicate a stronger

Table 1
Brain regions showing a significant ($p < .05$, FWE-corrected) main effect of the emotional face matching task, across groups and separately per session.

Region	Cluster size	Hemisphere	MNI coordinates			*T*-value
			x	y	z	
Session 1						
Posterior	3688					
inferior occipital gyrus		L	−24	−96	−6	16.54
inferior occipital gyrus		R	30	−87	−9	15.86
cerebellum, hemispheric lobule VI		L	−36	−51	−24	14.86
Frontal	907					
inferior frontal gyrus, triangular part		L	−45	24	21	10.48
inferior frontal gyrus, triangular part		L	−57	33	12	10.05
inferior frontal gyrus, triangular part		L	−54	39	6	9.05
Frontal	858					
inferior frontal gyrus, triangular part		R	39	18	21	11.59
inferior frontal gyrus, triangular part		R	54	33	18	9.87
middle frontal gyrus		R	51	3	54	7.73
Subcortical	452					
amygdala		L	−24	−3	−21	10.82
hippocampus		L	−21	−30	−9	7.95
temporal pole, superior temporal gyrus		L	−45	15	−21	6.59
Temporal	363					
middle temporal gyrus		R	54	−45	9	8.07
middle temporal gyrus		R	45	−63	15	7.33
Frontal						
supplementary motor area	113	R	3	18	57	7.09
Temporal						
middle temporal gyrus	83	L	−51	−45	6	6.63
Session 2						
Occipital	4612					
middle occipital gyrus		L	−21	−96	−3	19.73
middle occipital gyrus		R	33	−93	0	19.02
inferior occipital gyrus		R	30	−84	−9	18.73
Frontal	669					
inferior frontal gyrus, triangular part		R	45	24	21	11.58
inferior frontal gyrus, triangular part		R	57	30	9	10.43
middle frontal gyrus		R	51	9	51	8.66
Frontal	667					
inferior frontal gyrus, triangular part		L	−45	18	21	11.04
inferior frontal gyrus, triangular part		L	−51	30	9	9.69
inferior frontal gyrus, orbital part		L	−30	27	−6	6.52
Temporal	260					
superior temporal gyrus		R	45	−39	6	8.02
middle temporal gyrus		R	36	−48	12	7.29
middle temporal gyrus		R	45	−60	18	6.05
Frontal						
supplementary motor area	97	R	0	21	51	7.93
Parietal						
precuneus	53	R	6	−51	45	8.66

MNI = Montreal Neurological Institute space; *R = right; L = left.*

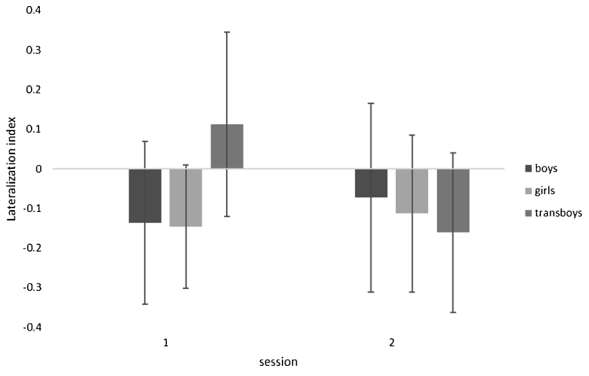

Fig. 4. The mean lateralization index of amygdala activation during emotional face processing per group per session. Positive lateralization values indicate leftward asymmetry, and negative lateralization values indicate rightward asymmetry. Error bars represent the 95% confidence intervals.

effect of testosterone after suppression as compared to natural change during puberty, possibly due to greater sensitivity of the receptors, or due to larger difference of testosterone levels between sessions in this group.

Focusing on the amygdala as our region of interest, in the first session we found that trans boys tended to show weaker functional amygdala lateralization compared to both, cisgender boys and girls. In addition, there were no significant sex differences in lateralization between the control groups. Our hypotheses therefore were only partly confirmed. After testosterone treatment, in line with the prediction, the lateralization of amygdala activation of trans boys changed significantly and became similar to that of cis boys. However, at the same time, at session 2 none of the three groups differed significantly from each other.

There was a trend for an interaction effect between session and group on change in amygdala lateralization, from session 1 to 2 which tended to be larger in trans boys versus cis boys, and trans boys versus cis girls. Of course, we should take these findings with some caution as the overall ANOVA did not reach statistical significance, perhaps due to a relatively small sample size and other reasons mentioned below. In

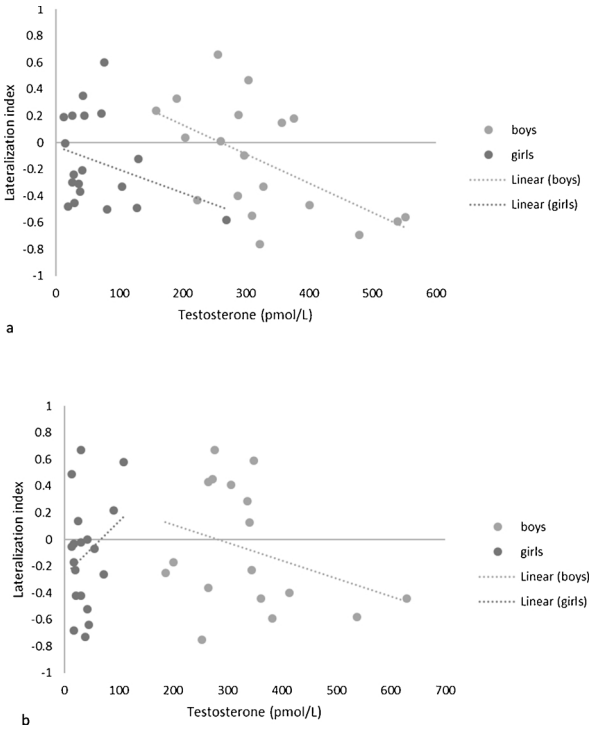

Fig. 5. The relation between testosterone and lateralization of amygdala activation during emotional face processing for the cis boys and cis girls for session 1 (5a) and session 2 (5b). Positive lateralization values indicate leftward asymmetry, and negative lateralization values indicate rightward asymmetry.

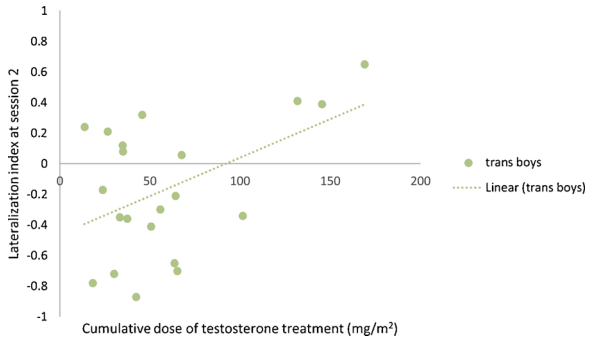

Fig. 6. The relation between cumulative dose of testosterone and the lateralization of emotional face processing for trans boys at session 2. Positive lateralization values indicate leftward asymmetry, and negative lateralization values indicate rightward asymmetry.

line with the literature, our findings tentatively suggest that testosterone treatment in trans boys may shift the lateralization of amygdala activation to the right to a level that is comparable to that in cis boys.

Pre-treatment lateralization in trans boys was not in between that of the control girls and control boys, as suggested by Cohen-Kettenis et al. (1998). In addition, we did find a rightward shift of lateralization for emotional face processing after about 10 months of treatment, whereas Sommer et al. (2008) report no effects of 3 months treatment on lateralization in other cognitive tasks (language and mental rotation). Given the marginal p-values of our findings we should, however, interpret the data with caution and replication with larger sample sizes and a more controlled design is certainly needed.

Next, we tested the relationship between the actual testosterone levels and amygdala lateralization in the cisgender control groups. The hypothesis that there would be a relation between endogenous testosterone and rightward functional amygdala lateralization was confirmed, but only for the cis boys at session 1. This outcome is in line with a study in adult men, finding a positive relation between endogenous testosterone levels and activation in the right, but not the left, ventral amygdala during emotional memory (Ackermann et al., 2012). Other studies found that testosterone levels were related to a bilateral increase (Manuck et al., 2010; Derntl et al., 2009) or decrease (Stanton et al., 2009) in amygdala activation while viewing emotional faces. This is not necessarily in contrast with our outcome, as these studies did not take the relative difference between the left and right amygdala into account as we calculated by means of the lateralization index. However, our finding that the lateralization index of cis girls – having lower testosterone concentrations – was comparable to that of cis boys at both sessions, indicates that genetic, other hormonal and/or environmental factors play an important role as well in the development of sex differences in lateralization (Arnold and McCarthy, 2016). The absence of a significant correlation between endogenous testosterone levels and amygdala lateralization in cis girls is likely to be due to the low testosterone levels with a limited range of values, and in line with the absence of such relationships in females in the studies of Ackermann et al. (2012) and Stanton et al. (2009).

Finally, we investigated the relation between exogenous testosterone concentrations at session 2 and amygdala lateralization in trans boys. We found no significant associations between circulating testosterone levels at session 2, but found that the cumulative testosterone treatment dose significantly predicted amygdala lateralization in the trans boys at session 2.

We propose the following explanations for these findings. Firstly, the actual testosterone levels as measured in trans boys at session 2 may not accurately reflect the testosterone treatment. This may be because testosterone levels fluctuate after the injection, starting with a supraphysiological peak and gradually decreasing over time (Bui et al., 2013). In addition, the cumulative dose of testosterone treatment differed between individuals, depending on the starting age and time between sessions. Therefore, the cumulative dose of exogenous testosterone may better explain the rightward shift in amygdala lateralization, reflecting a slow/developmental process rather than a current state, that might not have been completed by every trans boy participating in our study.

Secondly, there might be a neurobiological difference between trans boys and cis boys, explaining why there is a correlation between testosterone levels and lateralization in cis boys but not in trans boys. First of all, there might be differences between the effects of endogenous and exogenous testosterone. Also, trans boys might have a lower threshold for the effect of testosterone on lateralization than cis boys, and concentrations of testosterone in trans boys could have been above this threshold with testosterone treatment resulting in a ceiling effect. Genetic thresholding or ceiling mechanisms on the action of sex hormones are well known in both sexes, such as proteins that prevent dimerization or promote receptor translocation to the nucleus, or microRNAs that prevent translation of mRNA into protein (e.g., McCarthy, 2016).

Lastly, there might be a difference in the asymmetrical organization of the brain between trans boys and cis boys, influencing the effect of testosterone (Ernst et al., 2007). From human studies we know that there might be a sex difference in structural amygdala asymmetry from infancy to young adulthood (N = 109, Uematsu et al., 2012). In addition, earlier studies indicate that there might be sex differences in connectivity and in neuron size: in men functional connectivity with other brain regions was reported to be more widespread from the right amygdala, but in women from the left amygdala (Kilpatrick et al., 2006; Savic and Lindström, 2008), and neuron size was found to be larger in the right amygdala in men and in the left amygdala in women (Antyukhov, 2016), which is probably under the influence of androgens (Morris et al., 2008). If the structural lateralization of the amygdala differs between trans boys and cis boys, or if the androgen receptor distribution differs, then this might explain the different effects of testosterone in both groups.

4.1. Strengths, limitations, and future directions

So far, literature reporting sex differences in lateralization of amygdala activation was inconclusive, possibly because the analyses were performed per hemisphere, not taking the difference between both hemispheres into account. The strength of our study is that we determined a lateralization index for the amygdala. Moreover, previous human studies investigated the effects of endogenous testosterone in men, or single testosterone administration studies in women, on amygdala activity. For the first time, we investigated the effect of long-term exogenous testosterone treatment in trans boys and compared this with the effects of endogenous testosterone levels in control cisgender boys and girls, on amygdala lateralization.

There are several limitations of the present study. Apart from a relatively small sample size, there are a number of possible confounders that were not or poorly controlled for, such as circannual or monthly rhythms of testosterone levels and variability related to salivary testosterone sampling. Testosterone levels in girls at session 1 were very low, which may also be related to oral contraceptive use. Another limitation may be that saliva testosterone levels are only a proxy for testosterone concentrations that are metabolised in the brain. In addition, information on other sex hormones and sex hormone binding globulin (SHBG) that may modulate the relation between testosterone and the lateralization index should be investigated. A further limitation is that the trans boys received puberty suppression at session 1, and therefore we cannot distinguish if the stronger lateralization at session 2 is due to higher testosterone levels at that session, or due to lower lateralization scores at session 1 as a result of puberty suppression, or both. In addition, it would be of interest to examine and control for variations in gender identity in the control groups, e.g. by using a gender identity questionnaire in future studies.

The distinction between activating and organizing effects may not be as strict and are often difficult to disentangle. One study in trans individuals on hormone therapy suggests that hormones may play a role in right hemispheric cognitive processing (Cohen and Forget, 1995), but another study did not find indications for associations between endocrine measures nor atypical sexual differentiation in lateralization measures in trans women (Wisniewski et al., 2005). Because both studies were performed in trans people on hormone treatment without a measurement before start of that treatment, it remains unclear whether the findings are explained by their condition, the treatment, or both. In the present study, we also cannot distinguish between potentially activating and organizing effects of testosterone. Activating effects on amygdala function have previously been demonstrated in single testosterone administration studies in women (Bos et al., 2013; Hermans et al., 2008; van Wingen et al., 2009). Organizing effects of testosterone have been found on brain structure and function – including the amygdala – in puberty (Bramen et al., 2011; Goddings et al., 2014; Neufang et al., 2009; Sisk and Zehr, 2005). Ideally, future studies should use a more extensive longitudinal approach with multiple measurements in persons diagnosed with gender dysphoria. For example, in pre-pubertal children, in adolescents just before puberty suppression starts, and at several time points after the onset of treatment. These are the first steps to disentangle whether testosterone has activating and/or organizing effects on amygdala lateralization. In addition, in our study it is impossible to unravel activating from organizing effects since we have no measurements during withdrawal of testosterone. If the effects of testosterone would disappear during withdrawal it would be activating effects, when they remain, they would be organizational. Another future endeavor would be to investigate the effects of testosterone treatment in adult trans men, to determine the possible presence of a sensitive window for the hormonal effects. In addition, it would certainly be interesting as a next step to investigate amygdala to frontal functional brain connectivity changes due to the testosterone treatment in trans boys. A similar approach was employed by van Wingen et al. (2009), who found decreased coupling between the amygdala and orbito-frontal cortex after a single administration of testosterone to adult women, who performed an emotional face matching task.

An interesting addition would be to investigate the effect of estradiol treatment, for example in trans girls (male sex assigned at birth, female gender identity), which might also influence lateralization of the amygdala. Both testosterone and estradiol have been found to increase right amygdala growth across adolescence in both sexes (Herting et al., 2014), and effects of menstrual cycle on lateralized amygdala activation have been reported (Derntl et al., 2008). In addition, it is important to realize that testosterone and estradiol interact with each other, and that testosterone can be converted to estradiol by aromatase, which is highly prevalent in the amygdala (Pareto et al., 2004). Unfortunately, the difference in aromatase levels between the left and the right amygdala is, to the best of our knowledge, not known. In mice, the aromatization of testosterone to estradiol is essential for its effect on the number of neurons in the amygdala in puberty (Sano et al., 2016). In humans, it is unknown if testosterone directly acts on the amygdala, but it is generally assumed that testosterone *directly* affects sexual differentiation of the brain (Wallen, 2005).

4.2. Conclusions

The functional lateralization of the amygdala, and the influence of testosterone on this lateralization, has been a topic of debate for a long time. In the present study we tried to bridge the gap between experimental animal studies and correlational human studies, by investigating the effect of long-term testosterone treatment in trans boys. Our overarching hypothesis that testosterone predicts rightward lateralization of the amygdala was partially confirmed. In favor of this hypothesis were the findings that functional lateralization in trans boys tended to shift towards the right amygdala after testosterone treatment, that endogenous testosterone concentrations predicted rightward amygdala lateralization in cisgender boys at session 1, and that the cumulative dose of exogenous testosterone predicted rightward amygdala lateralization in trans boys at session 2. However, against our expectations, cisgender girls had a similar functional amygdala lateralization as cis boys and trans boys at both sessions. To investigate whether the partly inconsistent findings can be explained by a biological difference between birth-assigned boys and girls, such as differences in testosterone sensitivity, estrogen levels, or neurobiology, is open for future studies.

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Declaration of Competing Interest

None.

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