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Research report

Changes in spatial cognition and brain activity after a single dose of testosterone in healthy women

Carl W.S. Pintzka[a,c,*], Hallvard R. Evensmoen[a], Hanne Lehn[b], Asta K. Håberg[a,c]

[a] Department of Neuroscience, Norwegian University of Science and Technology (NTNU), 7489 Trondheim, Norway
[b] Department of Circulation and Medical Imaging, Norwegian University of Science and Technology (NTNU), 7489 Trondheim, Norway
[c] Department of Medical Imaging, St. Olav's Hospital, 7489 Trondheim, Norway

HIGHLIGHTS

- We administered testosterone in a randomized, placebo-controlled design to 42 women.
- We performed fMRI during wayfinding tasks in a recently learned virtual environment.
- Testosterone improved some aspects of spatial abilities in women.
- Testosterone increased medial temporal lobe activity during virtual navigation.

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ABSTRACT

Studies have consistently shown that males perform better than females on several spatial tasks. Animal and human literature suggests that sex hormones have an important role in both establishing and maintaining this difference. The aim of the present study was to examine the effects of exogenous testosterone on spatial cognition and brain activity in healthy women. A cross-sectional, double-blind, randomized, placebo-controlled study was performed in 42 healthy young women who either received one dose of 0.5 mg sublingual testosterone or placebo. They then learned a virtual environment and performed navigation tasks during functional magnetic resonance imaging (fMRI). Subsequently, their knowledge of the virtual environment, self-reported navigation strategy, and mental rotation abilities were measured. The testosterone group had improved representations of the directions within the environment and performed significantly better on the mental rotation task compared to the placebo group, but navigation success and navigation strategy were similar in the two groups. Nevertheless, the testosterone group had significantly increased activity within the medial temporal lobe during successful navigation compared to the placebo group, and a positive correlation between testosterone load and medial temporal lobe activity was found. Fetal testosterone levels, measured as second-to-fourth digit length ratio, interacted significantly with parahippocampal activity and tended towards giving higher mental rotation task scores. These results demonstrated that testosterone had a limited effect pertaining specifically to spatial cognition involving 3D-visualization in healthy women, while complex behaviors such as navigation, relying more on learned strategies, were not altered despite increased neuronal activity in relevant brain regions.

1. Introduction

Performance on certain cognitive tasks, such as spatial navigation and mental rotation tasks (MRT), is unevenly distributed between men and women. Indeed, MRT and navigation are the two tasks with the most consistent and notable sex differences with men scoring higher than women [1–6]. Furthermore, performance on MRT is moderately correlated to navigation ability [7,8]. The underlying biological mechanism(s) underpinning this sexual dimorphic performance remain uncertain, but several lines of evidence point to testosterone levels playing a significant role. In general, increased testosterone levels are associated with improved MRT performance in women [9–11]. More ambiguous results are found for navigation using a virtual analogue of the Morris water

* Corresponding author at: Department of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), 7489 Trondheim, Norway. Fax: +47 73551350.
E-mail address: carl.pintzka@ntnu.no (C.W.S. Pintzka).

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task (vMWT), with one study indicating a positive association between endogenous testosterone and navigation performance in women [8], and other studies showing a neutral [7] or negative association [12]. Testosterone has both organizational and activational effects on the brain, which may both be involved in the emergence of sexual dimorphic performance [13]. The organizational effect takes place during development and results in permanent changes in brain morphology. Fetal testosterone levels are believed to be mirrored in the ratio between the length of the second and fourth digit (2D:4D) [14], which is used in some studies as a proxy for testosterone's organizational effects on the brain [15–19]. The activational effect of testosterone is more transient and depends on the current endogenous hormone level [20,21]. Both organizational and activational effects influence brain function, and interactions between organizational and activational effects are reported [22,23].

Navigation ability is subserved by several medial temporal lobe (MTL) structures [24], predominantly the hippocampus, entorhinal and parahippocampal cortices [25–28]. Both androgen and estrogen receptors are particularly densely expressed in the MTL [29,30], and sex hormones have been demonstrated to have major effects on hippocampal structure and function [8,31–33]. In the hippocampus, androgens can act on pyrmidal cells both directly via the androgen receptor, as well as via one of the estrogen receptors after convertion to estrogens by aromatase [34]. Activational effects of testosterone have been shown in women, as adult endogenous testosterone levels correlate positively with navigation abilities in both adult women and female rats [8,35]. In animal models, it has been shown that testosterone administration increases hippocampal pyramidal dendritic spine density in ovariectomized female rats [36]. Hippocampal pyramidal dendritic spine density is positively associated with memory [37–39], and thus possibly navigation performance. Moreover, women born with congenital adrenal hyperplasia, which is associated with excessive production of androgens in fetal life, perform better on spatial tasks than healthy controls [40] pointing to an organizational effect of testosterone on spatial cognition in women. Still, no study has assessed the effect of exogenous testosterone on navigation performance in women, nor examined possible interactions between testosterone's activational and organizational effects in navigation, and/or their separate contributions to navigation.

A positive effect of exogenous testosterone administration on MRT performance in women is, on the other hand, well documented [10,41,42]. In contrast to navigation, mental rotation is not dependent on the hippocampus, but relies on frontal and parietal regions [43]. Since there are no experimental animal models of mental rotation, less is known about the possible neurobiological mechanism(s) behind testosterone's MRT enhancing effect. However, sex hormone receptors are located throughout the cortex [44], including frontal and parietal regions, and numerous effects have been ascribed to sex hormone receptor activation [33,45–49]. At the activational level, it has been shown both that endogenous testosterone levels correlate positively with MRT performance [9], and that a single dose of exogenous testosterone improves MRT performance in healthy women [10]. Moreover, prolonged androgen treatment in female-to-male transsexuals improves MRT performance even several weeks after cessation of testosterone administration [41]. There is conflicting evidence with regard to testosterone's organizational effects on MRT performance [15,16,18,19], but women exposed to higher intrauterine testosterone levels, i.e., women with congenital adrenal hyperplasia and female fraternal twins with male co-twins, perform better than controls on several spatial tasks, including MRT [11,40,50,51].

The neuronal correlates of testosterone's effect on behavior in healthy women have so far only been studied with paradigms probing social interaction, reward and mood, and only by comparing overall responses to stimulus presentation versus a passive baseline condition. A general finding across these studies is increased brain activity in task positive regions [52–57]. However, whether this increase in activity is related specifically to task performance or reflect a general increase in the BOLD signal due to the mere exposure to a stimulus, cannot be surmised from these results. In addition to increased brain activity, both increased and decreased functional connectivity has been described during task performance [54,58,59], but no study has examined if exogenous testosterone influences resting state functional connectivity. The described effects of exogenous testosterone on brain activity have always been measured after a delay of several hours, when serum levels have returned to normal, pointing to genomic mechanisms behind the changes in fMRI activity [23,52–55,57,60].

The primary aim of the current study was to explore the effect of exogenous testosterone administration on navigation ability and its neuronal correlates in women using an ecologically valid, large scale virtual environment (VE). Based on the above reviewed literature, we predicted that sublingual administration of a single dose of testosterone would increase spatial navigation performance in women. Furthermore, we predicted that testosterone administration would improve knowledge of the environment, i.e., improve judgments of direction and distance between landmarks and increase use of available shortcuts. The latter prediction was motivated by previous studies showing that men perform better than women when navigation requires accurate directions [5,6,61], have better pointing and navigation accuracy towards unseen targets [62,63], and better distance judgment compared to women [62,64].

We further predicted that testosterone administration induced changes in brain activity in certain regions of the MTL, and that these changes would be specific to successful navigation. Specifically, we predicted increased activity in the anterior hippocampus in the testosterone group, as activity in this area has been linked to the use of an allocentric strategy, i.e. a more male like navigation style [65], during VE navigation [25], and correlates with successful VE navigation [26]. The overall activity during navigation compared to baseline, on the other hand, would be similar in the two groups. As a control, the effect of exogenous testosterone on MRT performance was assessed to ascertain that the experimental setup allowed for reproduction of the earlier described performance effects of testosterone. Since some previous studies suggest an interaction between current testosterone level and fetal testosterone exposure on task performance [22,23], we investigated correlations and interactions between the 2D:4D ratio, navigation performance, MRT performance, and brain activity in the MTL during successful navigation. Lastly, we explored the effects of exogenous testosterone on resting state networks based on previous reports on increased functional connectivity in task positive regions following testosterone administration.

2. Materials and methods

2.1. Participants

53 healthy right-handed women (19–30 years, mean = 22.5 years) were recruited at the university campus. The local ethics committee only allowed the enrollment of participants that were on oral contraceptives. Moreover, the participants had to take a pregnancy test at the day of the experiment to ensure that pregnant women were not included. To control for menstrual cycle induced hormonal fluctuations and to ensure similar low endogenous hormone levels in all participants, the women were instructed to stop using oral contraceptives three to seven days prior to the study to ensure that they were in their early follicular phase [66]. At this time

point women have the lowest estradiol levels. Several studies have found associations between endogenous estradiol levels and performance on spatial tasks in women [9,67,68]. Furthermore, lower estradiol levels will minimize potential competition for estrogen receptors between estradiol and testosterone [34]. The participants' computer gaming experience was assessed by asking if and what type of experience they had with playing 1st person computer games. None of the participants reported notable computer gaming experience. All participants provided written informed consent, and received 500 Norwegian kroner as reimbursement. The study was approved by the National Committee for Medical Research Ethics in Midt-Norge, Norway.

2.2. Virtual environment

The VE has been described in detail previously [25,26]. Briefly, the environment mimics the inside of one floor in a large-scale modern office building with rooms, corridors and open areas of various sizes, but lacking exterior windows. The current version of the environment included 76 landmarks inside and 6 landmarks outside the office building. The landmarks inside the office building were spread throughout the environment, and each of them consisted of a distinct group of objects and/or pictures. The landmarks outside the office building were skyscrapers visible to the participants through the glass ceiling and located in all cardinal directions (Fig. 1A).

2.3. Pre scanning

After inclusion, the participants were randomly divided in two groups. One group received 0.5 mg sublingual testosterone solution; the other received a sublingual placebo solution. This procedure was double blinded; both solutions tasted and looked identical, and were in identical containers labels A and B. Blood was drawn immediately before administration of testosterone/placebo, 30 min after and 100 min after. Serum concentrations of total testosterone, estradiol, progesterone, androstenedione, sex hormone-binding globulin (SHBG) and dehydroepiandrosterone sulfate (DHEAS) were analyzed.

The testosterone dose used in the current study was 0.5 mg sublingual cyclodextrine-bound testosterone (ACE Pharmaceuticals, Zeewolde, The Netherlands). This dose results in a 25-fold increase in serum testosterone (s-testosterone) concentration with T_{max} (time to maximum) at 15 min, followed by a slow return to baseline over 150 min [69]. Two hours after administration of placebo/testosterone, the participants began exploring and learning the VE using a desktop computer. They began by exploring freely for 20 min, followed by four structured navigation tasks of varying length to become familiarized with the VE in its full extent and to see all landmarks at least once. In each of these four tasks, the participants started at a specific landmark in the environment and had to find a target landmark that was shown at the bottom center of the screen. Upon arrival at the target landmark, a new target landmark was presented. The participants were given new target landmarks in the same manner until a whole sequence of landmarks was completed. The participants were excluded if they were unable to finish the four structured navigation tasks, which encompassed locating a total of 60 target landmarks within 90 min. Next, the fMRI paradigm was demonstrated, followed by a short break. fMRI scanning was commenced four hours after placebo/testosterone administration, since previous fMRI studies investigating the impact of exogenous testosterone on different cognitive and emotional measures have shown effects on both behavior and brain activity using this delay [10,23,52,54,60].

2.4. Scanning procedure

Scanning was performed on a 3T Siemens Trio scanner with a 32-channel Head Matrix Coil (Siemens AG, Erlangen, Germany). Foam pads were used to minimize head motion. The fMRI stimuli were presented on an LCD monitor with a resolution of 1280×1024, and the participant moved inside the virtual environment using an MRI compatible joystick (Current Designs, Philadelphia, US).

2.5. fMRI paradigm and resting state fMRI

The fMRI paradigm was a self-paced block design task with alternating blocks of navigation (max. duration 40 ± 2 s), and odd-even number judgments (15 s), the latter serving as the implicit baseline [70]. In the navigation condition participants were placed at a different landmark at the start of each block. An image of a target landmark was shown at the bottom center of the screen, and the participants were instructed to move as fast and accurately as possible to this landmark using the joystick (Fig. 1A). Navigating within the VE used in the current study has been reported to depend on MTL activity in several previous publications [25,26]. The baseline task was an odd-even task [70] which involved pushing the right joystick button when a random even number (<100) appeared and the left joystick button when a random odd number (<100) appeared. The participants completed three experimental runs, with 15 navigation blocks and 15 odd-even blocks in each run. The order of the blocks was randomized within each run for each participant, and the runs were randomized between participants. Position data of the participants' movements inside the environment were logged with a time interval of 30 ms. After completing the fMRI task, the participants also completed a resting state fMRI session during which they were instructed to relax and keep their eyes open for 8 min.

2.6. Imaging parameters

T2* weighted, blood-oxygen-level-dependent (BOLD) sensitive images were acquired during the navigation tasks using an echo-planar imaging (EPI) pulse sequence (TR = 2920 ms, TE = 28 ms, field of view (FOV) = 129 mm, slice thickness = 1.9 mm, slice number = 40, matrix = 68×68, giving a voxel size of $1.9 \times 1.9 \times 1.9$ mm). A restricted FOV was chosen in order to be able to acquire high-resolution scans of the MTL. In order to avoid fold in artifacts, oversampling and saturation slabs were used in the phase encoding direction. The slices were positioned as close to 90° on the anterior-posterior direction of the hippocampus as possible. GRAPPA acceleration was used with a factor four. Each functional run contained 258 ± 29 volumes. For distortion correction, two spin eco images (TR = 5810 ms, TE = 100 ms, FOV = 129 mm, slice thickness = 1.9 mm, slice number = 40, matrix = 68×68), in opposite phase encoding polarities (anterior–posterior and posterior-anterior), were acquired [71].

Whole brain resting state fMRI was acquired using an EPI pulse sequence (TR = 2880 ms, TE = 28 ms, FOV = 238 mm, slice thickness = 2.6 mm, slice number = 44, matrix = 90×90). A total of 180 volumes were acquired over 8 min.

For anatomical reference a T1 weighted (T1W) 3D volume was acquired with an MPRage sequence (TR = 1900 ms, TE = 2.32 ms, FOV = 230 mm, slice thickness = 0.9 mm, matrix 256×256). In order to optimize the registration of the restricted FOV BOLD series, a T2* weighted whole brain image using an EPI pulse sequence (TR = 6140 ms, TE = 28 ms, FOV = 238 mm, slice thickness = 1.9 mm, slice number = 80, matrix = 128×128), was acquired. For distortion correction of the T2* whole brain image, two spin eco images (TR = 10,990 ms, TE = 100 ms, FOV = 238 mm, slice thickness = 1.9 mm, slice number = 80, matrix = 128×128),

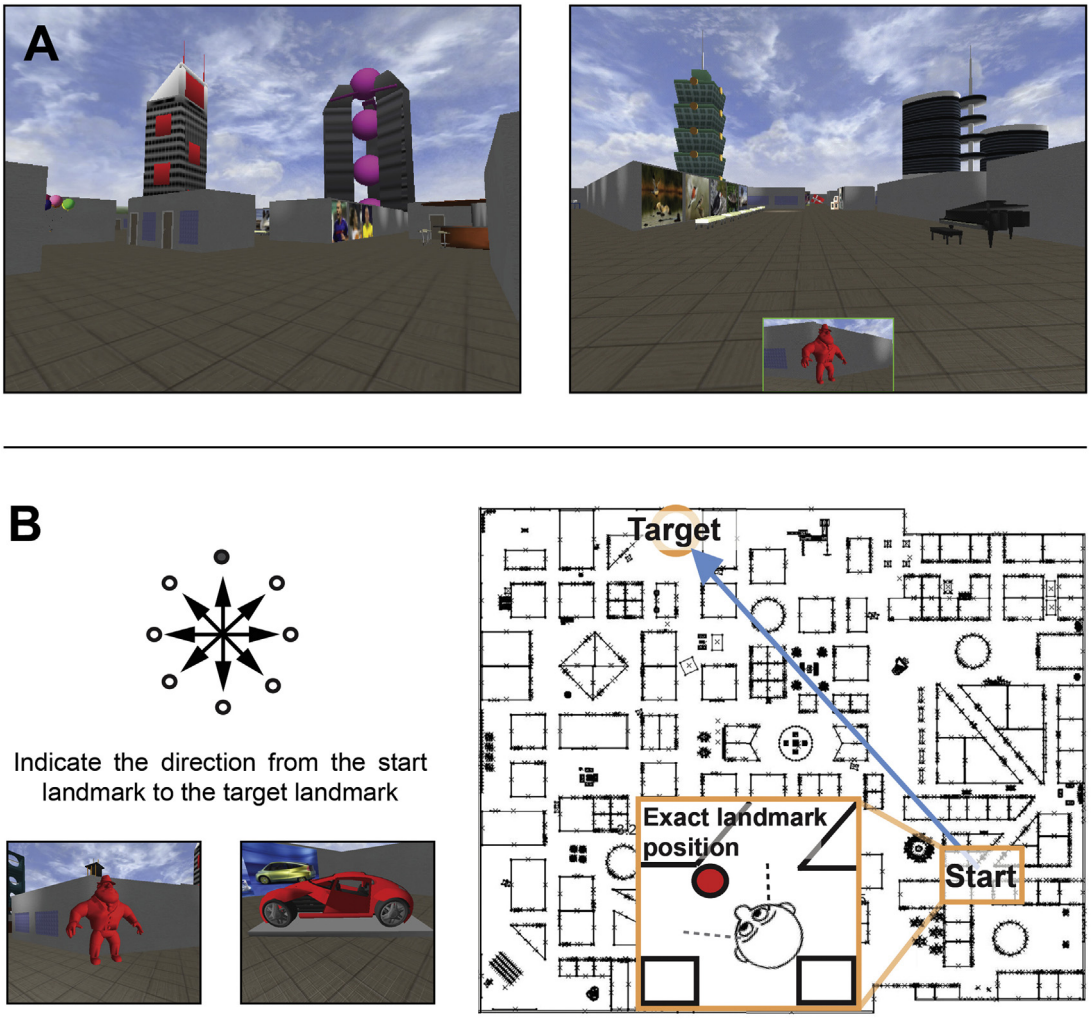

Fig. 1. The virtual environment and direction task.
(A) Screenshots from two different areas within the virtual environment (VE). The left image shows a part of the VE as seen during the free exploration period. In the right image the navigation target is presented at the bottom of the screen. (B) The left part illustrates a VE direction task. Two points were given for correct direction and one was given for the two adjacent directions. The right part illustrates the task on a 2D overview of the entire VE (not shown to the participants). The correct choice in this case is the arrow straight ahead.

in opposite phase encoding polarities (anterior–posterior and posterior–anterior), were acquired.

2.7. Post-scanning

After scanning the participants completed a questionnaire aimed at assessing the use of allocentric and egocentric strategies when navigating through the environment. To this end we used a study specific strategy questionnaire (SSSQ) inspired by the *sense of direction questionnaire-short form* [72], but pertaining specifically to the current VE. The SSSQ consists of 24 questions, eight of which assess the use of allocentric and egocentric navigation strategies and were used in the current study. These eight questions were; "At the start of a task, when I was placed at a random location within the VE, I located my position using.": (1) " a 2D map-like representation of all the outside landmarks", (2) "...a 2D map-like representation of the inside landmarks", (3) "... where I was in a sequence of inside landmarks", (4) "...where I was in a spatial route of inside landmarks", and "When I was planning where to go to the target landmark, I used...": (5) "...a 2D map-like representation of all the outside landmarks", (6) "...a 2D map-like representation of the inside landmarks", (7) "... where I was in a sequence of inside landmarks", (8) "...where I was in a spatial route of inside landmarks". The participants rated the questions from strongly agree (9) to strongly disagree (1). Questions 1, 2, 5 and 6 entail the use of a map-like representation of the environment and thus represent the use of an allocentric (world-centered) strategy [73], whereas questions 3, 4, 7 and 8 entail the use of a sequence/route of landmarks and thus represent the use of an egocentric (self-centered) strategy [73]. Scoring on questions 3, 4, 7 and 8 were inverted; a high total score therefore represents an allocentric strategy whereas a low total score represents an egocentric strategy. In general, men rely predominantly on an allocentric strategy when orienting themselves in an environment, whereas women more often use an egocentric strategy [65,74]. Allocentric navigation is considered to rely on structures in the MTL, predominantly the hippocampus, and the parahippocampal and posterior entorhinal cortices [24,27,28,75–78]. Egocentric navigation, on the other hand, is considered to depend on the striatum [28,79–85], although human and animal studies also suggest a role for the hippocampus [24,27,28,75,76].

After completing the SSSQ, participants were asked to rate their overall level of nausea during the VE experience on a scale from zero to ten. During VE navigation, participants are exposed to a moving landscape without appropriate physical motion, which is known to cause nausea due to the illusion of self-motion in the opposite

direction [86]. There have been reports of more women than men experiencing nausea during virtual navigation [87].

Subsequently the participants performed three computer-based tests to ascertain their level of proficiency of the virtual environment [25,26]: (1) recognition of landmarks, where they were asked to pick out 15 landmarks that were inside the environment from a collection of 30 landmarks. (2) Judgment of distance between landmarks, where they in each task were asked to pick out a landmark closest to a reference landmark from a collection of four. (3) Judgment of direction between landmarks, where they in each task were asked in what direction a target landmark was from a reference landmark. There were eight alternatives based on north-south and east-west directions. Two points were given for indicating the correct direction and one point was given for indicating either of the two adjacent directions (Fig. 1B).

2.7.1. 2D:4D measurements

Since the 2D:4D ratio may be a proxy for fetal testosterone effects that in turn may have implications for adult performance on cognitive tasks, this factor needs to be taken into consideration when examining hormonal effects on cognition. Therefore, digital copies of all participants' right hand were taken, and measurements of the 2nd and 4th digit length were made using Photoshop (Adobe systems, San Jose, USA). All digits were measured by two independent investigators, mean value reported.

2.7.2. Mental rotation

The Vandenberg and Kuse (VK) [3] version of the MRT is considered to be the cognitive test most sensitive to sex differences, as well as levels of sex hormones [2]. A digital version of the VK MRT was administered at the end of the experiment, approximately five hours after the testosterone/placebo administration. The participants used a desktop computer and were presented with an image of a 3D-object built of 10 cubes. Below this object four smaller objects of similar construction were displayed. Two of these were correct alternatives and two were incorrect distractors. The two correct objects were rotated versions of the main object; the distractors were rotated mirror-objects of the main object. The test consisted of 20 problems and the participants had 5 min to solve as many of these problems as possible, since the magnitude of sex difference in MRT has been shown to be linearly correlated to the amount of time available [88]. Scoring is done by giving 1 point if both choices are correct and to give no credit otherwise. This eliminates the need to correct for guessing.

2.8. Data analysis

2.8.1. Hormone analysis

Hormone analyses were performed at the Department of Clinical Chemistry at St. Olav's University Hospital in Trondheim, Norway. Serum concentrations of total testosterone and androstenedione were measured by liquid chromatography separation followed by tandem mass spectrometry (LC/MS–MS), using an Agilent 1290 chromatograph with an Agilent 6410 Triple Quad LC/MS–MS detector (Agilent, California, USA). Serum concentrations of estradiol, progesterone, SHBG and DHEAS were analyzed by electrochemiluminescence immunoassay (ECLI) in a Roche Modular E analyzer (Roche Diagnostics, Mannheim, Germany). The intra- and interassay coefficients of variation were 3.0 and 6.0% for testosterone, 3.2 and 5.0% for androstenedione, 3.8 and 3.8% for estradiol, 4.4 and 4.1% for progesterone, 2.3 and 2.4% for SHBG, and 3.7 and 4.8% for DHEAS, respectively. To estimate testosterone load, the area under the curve (AUC) for s-testosterone during the first 100 min was calculated using trapezoidal approximation. Statistically significant differences in hormone levels were identified using a mixed ANOVA model. A corrected p-value of <0.05 using the Benjamini-Hochberg method, which controls the false discovery rate, was considered significant [89].

2.8.2. Demographic and performance data analysis

Group differences in age, 2D:4D ratio, day in the menstrual cycle, testosterone load and nausea were investigated with independent-sample t-tests and not corrected for multiple comparisons, since avoiding type I errors when describing the two groups was the primary objective. A MANCOVA was run to examine the effects of drug group (placebo/testosterone) on the outcome measures, using 2D:4D as covariate to identify any potentiating effects of fetal testosterone levels. There were eight outcome measures; total learning time (time to complete the structured learning tasks), navigation success (percentage of tasks completed within the VE during fMRI), navigation success time (mean time to complete all successful fMRI navigation task), scores on the three post-scanning tests of landmark recognition, distance and direction, navigation strategy (SSSQ score) and finally MRT score. A corrected p-value of <0.05 using the Benjamini-Hochberg method was considered significant [89]. Pearson's product-moment correlations were calculated to assess the relationship between testosterone load and scores on the two tests where a group difference was found, MRT and VE direction, and between MRT score and navigation success. An uncorrected p-value of <0.05 was considered significant. All statistical data were analyzed in SPSS 21.0 (SPSS Inc., Chicago, Illinois, USA).

2.8.3. MRI data analysis

Distortion correction of the $T2^*$ weighted images was conducted using spin echo images with opposite polarities [71]. Imaging data were then analyzed using FSL 4.1.8 (Analysis Group, FMRIB, Oxford, UK). Non-brain tissue was removed from the T1W anatomical images using BET2 with robust center estimation (Brain Extraction Tool, FMRIB, Oxford, UK). The resulting images were transformed to the MNI $1 \times 1 \times 1$ mm template (Montreal Neurological Institute, Montreal, QC, Canada) nonlinearly with FNIRT (FMRIB, Oxford UK). The fMRI data was motion corrected using MCFLIRT, using the median volume of each run as reference. Next, each functional run was co-registered to the whole brain EPI image, and subsequently to the anatomical T1W image, before it was transformed into MNI space by using the transformation matrix obtained with the T1W image. The functional data was smoothed with a 3 mm full-width at half-maximum Gaussian filter, and temporally high-pass filtered with a cut-off time of 250 s.

2.8.4. fMRI data analysis

The statistical analysis of the fMRI data was carried out in FEAT (FEAT, FMRIB, Oxford, UK). Conditions were modeled according to a boxcar stimulus function convolved with a two-gamma hemodynamic response function. The effect of each condition was estimated with GLM using FLAME 1 (FMRIB's Local Analysis of Mixed Effects). A brain mask was applied to investigate activation in the MTL only. The mask was created from the probabilistic maps of the Harvard Oxford Structural Atlases (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Atlases), using no probability threshold. The mask contained 92,469 1 mm voxels, and included the left and right hippocampus, parahippocampal gyrus and amygdala. Effects of testosterone on brain activity during navigation were investigated by the contrasts navigation > baseline (odd-even) and successful > failed navigation to control for performance differences between the drug groups. In the latter, runs with only successful or only failed navigation trials were excluded (7% across both drug groups). Moreover, the contrast successful > failed navigation was run with 2D:4D as a confounder to investigate to what extent the drug group differences could be explained by fetal testosterone levels, whether there were any interactions between 2D:4D

Table 1
Concentrations of hormones in the two groups immediately before administration of testosterone/placebo, and after 30 and 100 min.

	Testosterone ($n = 21$)			Placebo ($n = 21$)		
	Baseline	30 min	100 min	Baseline	30 min	100 min
Estradiol (nmol/l)	0.07 ± 0.06	0.07 ± 0.06	0.08 ± 0.07	0.09 ± 0.06	0.09 ± 0.06	0.08 ± 0.07
Progesterone (nmol/l)	1.24 ± 0.44	1.27 ± 0.48	1.14 ± 0.50	1.22 ± 0.51	1.11 ± 0.48	1.07 ± 0.48
SHBG (nmol/l)	207.9 ± 106	205.1 ± 104	200.5 ± 105	181.5 ± 74	180.7 ± 77	185.2 ± 73
Androstendione (nmol/l)	7.03 ± 3.55	7.29 ± 3.10	5.46 ± 3.10	7.51 ± 3.03	5.66 ± 2.30	5.68 ± 2.37
DHEAS (μmol/l)	4.44 ± 2.05	4.37 ± 2.02	4.44 ± 2.11	4.81 ± 2.01	4.65 ± 1.88	4.57 ± 1.85
Testosterone (nmol/l)	1.14 ± 0.44	23.20 ± 7.83*	3.17 ± 0.93*	1.15 ± 0.45	0.99 ± 0.42*	1.04 ± 0.39*

SHBG: sex hormone-binding globulin. DHEAS: dehydroepiandrosterone sulfate. Data are presented as mean ± SD.
* Main effect of group, corrected $p < .001$.

and drug group, and finally whether there were any areas in the MTL where the BOLD signal correlated with 2D:4D. Further, an analysis with testosterone load as a separate regressor was run for all participants combined to identify possible associations between the BOLD signal in MTL and the testosterone load. Independent samples t-tests were used to test for drug group differences in MTL activity. A threshold of $Z > 2.3$, corrected for multiple comparisons using cluster-extent tresholding [90] at $p < 0.05$ was used.

2.8.5. Resting state fMRI analysis

The resting state fMRI datasets contained 180 time points for each participant and were preprocessed as described in Section 2.8.3. The preprocessed data were temporally concatenated across participants to create a single 4D dataset using MELODIC. Thirty independent components were extracted, each representing a network of brain regions with correlating BOLD signal time courses. The dual regression method [91] was used to estimate the correspondence between each of these components and the individual participant data. Group differences were tested with non-parametric permutations as implemented in FSL's randomise tool, controlling for the family-wise error rate using threshold-free cluster enhancement at $p < .05$ [92,93].

3. Results

3.1. Participants

Seven (three in testosterone group) of the 53 included participants were unable to finish the VE learning period due to excessive nausea, and these participants were therefore excluded from further participation. Of the 46 remaining women, four (two in the testosterone group) were excluded for failing to complete the structured learning task in the VE within the predefined 90 min time limit. Only results from the 42 participants (21 in each drug group) who completed the entire experiment were included in the analyses. There were no differences in age (23.2 ± 2.9 vs. 21.8 ± 2.2 years; $p = .097$) or 2D:4D ratio (.973 ± .025 vs. .966 ± .019; $p = .333$) between the two drug groups.

3.2. Hormonal measurements

All participants were in the early follicular phase of their menstrual cycle (day 2.1 ± 1.2 vs. 2.3 ± 1.1; $p = .596$) at the time of the experiment, verified by the hormonal measurements (Table 1). There were no differences in the baseline levels of the different hormones or sex-binding protein between the two drug groups (all $p > 0.05$). The sublingual testosterone solution increased the serum concentration of testosterone 20-fold (drug group × time interaction, $F(2,70) = 143.6$, corrected $p < .001$). There was a statistically significant difference in s-testosterone at 30 min, $F(1,40) = 168.3$, $p < .001$, and at 100 min, $F(1,40) = 84.0$, corrected $p < .001$, resulting in significantly higher testosterone load in the testosterone compared to the placebo group (1150.0 ± 367.3 vs. 93.1 ± 37.7 nmol/l/min; corrected $p < .001$). Testosterone administration did not significantly change the concentrations of androstenedione, estradiol, progesterone, SHBG and DHEAS (all drug group × time interactions, $p > .05$).

Table 2
Scores on tests and behavior in the virtual environment.

	Testosterone	Placebo	p-value
Learning time (s)	2628 ± 1300	2819 ± 1077	1.000
Navigation success (%)	77.0 ± 15.7	74.6 ± 15.0	1.000
Navigation success time (s)	27.9 ± 4.5	27.0 ± 5.6	1.000
Object recognition (%)	98.6 ± 2.2	99.2 ± 1.5	1.000
Object distance (%)	67.6 ± 20.7	59.0 ± 19.7	.743
Object direction (%)	54.3 ± 13.0	40.7 ± 12.7	.029*

Learning time: Total learning time in seconds. Navigation success: Percentage of tasks completed within the environment. Navigation success time: mean time to complete a task in seconds. Object recognition: Percentage of objects recognized correctly in the recognition task. Object distance: Percentage of correct answers on the distance-test. Object direction: Percentage of correct answers on the direction-test. Data are analyzed using ANCOVAs with 2D:4D as covariate and are presented as mean ± SD. *Corrected p-value < .05.

3.3. Task performance, strategy, nausea and impact of 2D:4D on task performance

There were no interactions between drug group and 2D:4D ratio on any of the performance measures, thus only the main effects of drug group is reported.

3.3.1. Pre scanning VE learning time

The VE learning time was similar in the two drug groups (Table 2).

3.3.2. Behavior and nausea during fMRI

The testosterone and placebo groups had similar navigation success rate, $F(1,39) = .084$, corrected $p > .05$, partial $\eta^2 = .002$, and mean time to complete the tasks, $F(1,39) = .292$, corrected $p > .05$, partial $\eta^2 = .007$ (Table 2). The placebo group reported being significantly more nauseous during navigation (5.3 ± 3.4 vs. 2.8 ± 3.0 on a scale from 1 to 10; $p = .016$, $d = .78$).

3.3.3. Post scanning questionnaire, virtual environment knowledge tests, MRT performance and 2D:4D ratio

The strategy used during navigation was predominantly egocentric, and did not differ between the two drug groups based on the SSSQ, $F(1,39) = 0.489$, corrected $p > .05$, partial $\eta^2 = .012$. The testosterone group performed better on the VE direction test $F(1,39) = 9.785$, corrected $p = .029$, partial $\eta^2 = .201$ (Fig. 1, Table 2). There were no differences between the two drug groups in performance on the VE landmark recognition and distance tests. Finally, the testosterone group performed significantly better on the MRT

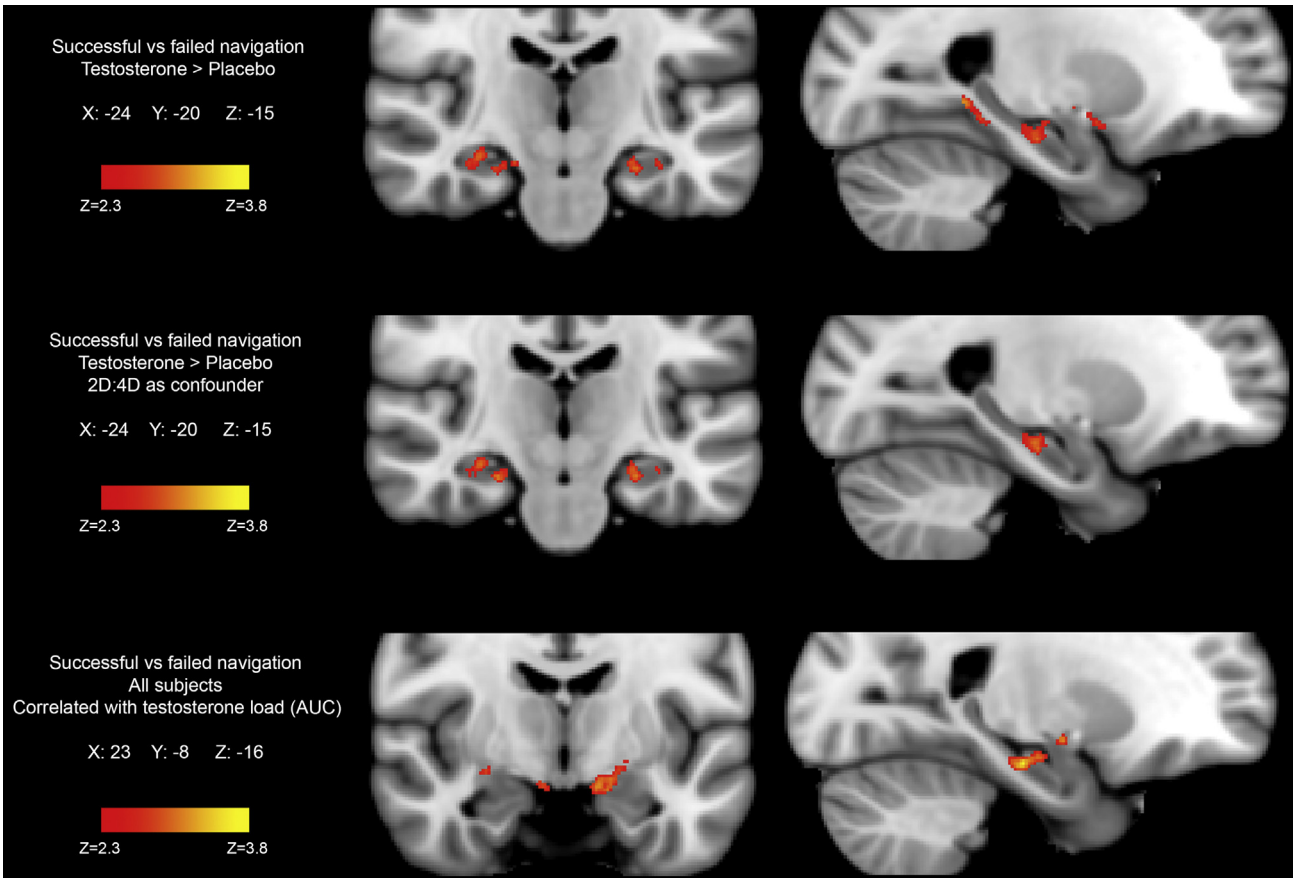

Fig. 2. fMRI activity during navigation.
Top: Increased activation in the medial temporal lobe (MTL) during successful navigation in the testosterone group. Middle: Increased activation in the anterior MTL during successful navigation in the testosterone group, after controlling for fetal testosterone levels (2D:4D ratio). Bottom: MTL activity correlated with testosterone load for all participants combined. All analyses were carried out using the MTL mask and $Z > 2.3$, cluster-corrected at $p < 0.05$. AUC: Area under the curve.

than the placebo group $F(1,39) = 7.911$, corrected $p = .032$, partial $\eta^2 = .169$ (Fig. 3), even if both drug groups completed the same number of MRT tasks within the 5-min time frame (16.8 ± 2.7 vs. 17.0 ± 3.4; $p = 0.83$).

The 2D:4D ratio did not correlate with navigation success, VE direction test or MRT scores in neither the testosterone nor the placebo group. No significant correlation was found between 2D:4D and total score on the SSSQ in any of the drug groups. For illustrative purposes, all participants, and subsequently the testosterone and the placebo groups, were dichotomized into a high and a low 2D:4D group by using the median 2D:4D ratio. There was no significant difference between the group of all participants with high 2D:4D ratio (low fetal testosterone) and the group with low 2D:4D ratio group (high fetal testosterone) on MRT performance, and no correlation between 2D:4D ratio and MRT scores. Nonetheless, a trend was present in both the placebo and the testosterone group, where women with low 2D:4D ratio scored higher on the MRT than their counterparts (Fig. 3).

3.4. Correlations between performance data and testosterone load across all participants

There was a moderate positive correlation between testosterone load and MRT score ($r = .328$, $p = .042$) and between testosterone load and the direction task score ($r = .429$, $p = .009$). A moderate, non-significant positive correlation was found between navigation success and MRT score ($r = .312$, $p = .053$).

3.5. fMRI activation differences in the anatomical MTL ROI

3.5.1. Between group differences for contrast navigation > baseline

There were no significant differences in activity within the anatomical MTL ROI between the two drug groups.

3.5.2. Between group difference for contrast successful > failed navigation

The testosterone group had significantly increased activity in the parahippocampal cortex, hippocampus and amygdala bilaterally in the contrast successful > failed navigation. The placebo group had no areas of increased activity compared to the testosterone group within the MTL mask (Fig. 2, Table 3).

3.5.3. Between group difference for contrast successful > failed navigation with 2D:4D as confounder and correlations between 2D:4D and BOLD signal for contrast successful > failed navigation across all participants

Adding 2D:4D as a confounder in the analysis did not change the activity found in the hippocampus or amygdala, however, the parahippocampal activity disappeared bilaterally. Again, the placebo group had no areas of increased activity compared to the testosterone group within the MTL mask (Fig. 2, Table 3). No significant interactions between 2D:4D and drug group was found, indicating a similar effect of exogenous testosterone, regardless of fetal levels. Moreover, no significant correlations between 2D:4D

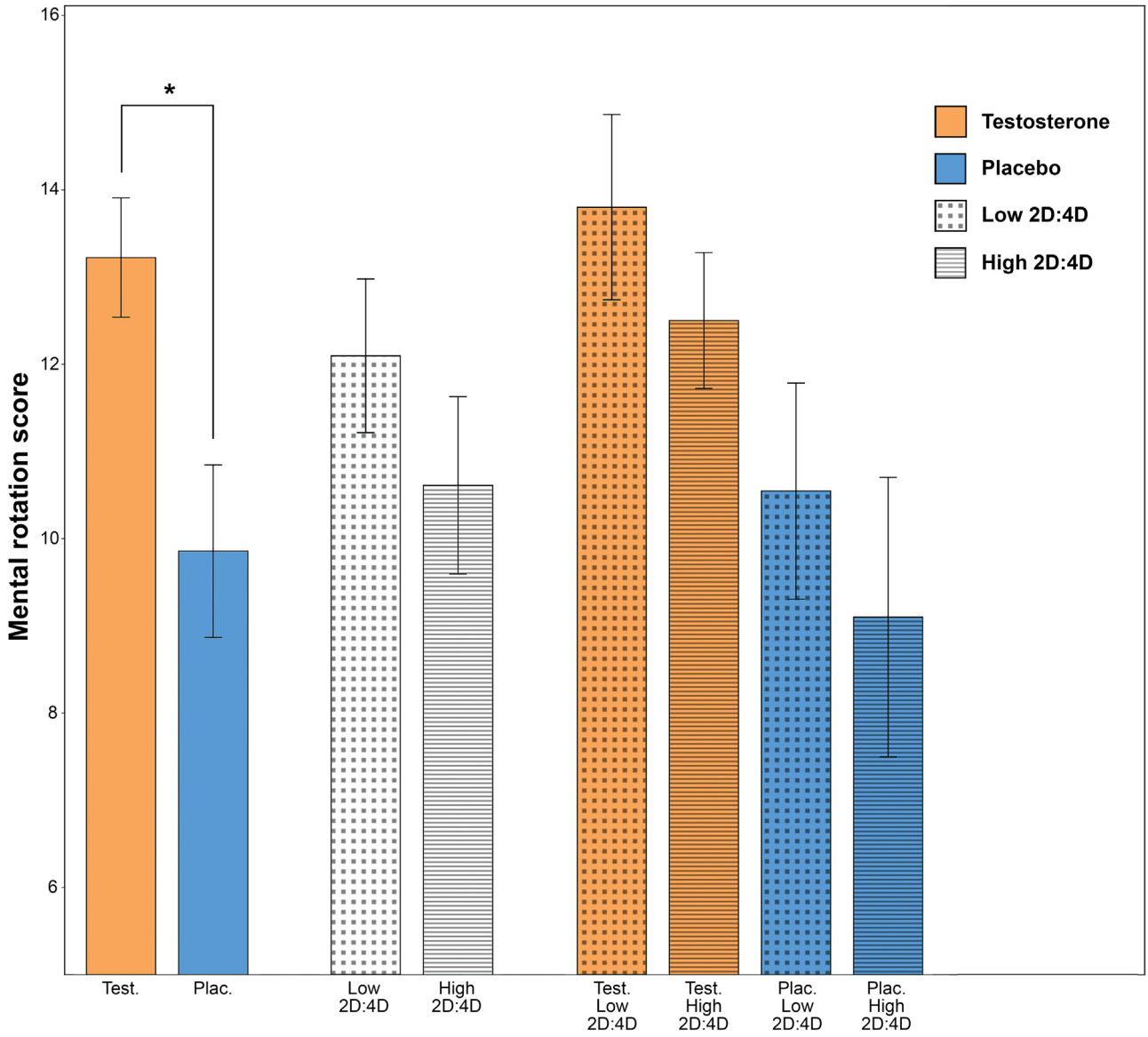

Fig. 3. Mental rotation task (MRT) scores varied according to both administered testosterone and fetal testosterone levels, as measured with 2D:4D.
The testosterone group had a significant higher MRT score than the placebo group. There was a non-significant trend towards a positive effect of fetal testosterone on MRT scores. Test. = testosterone group. Plac. = placebo group. Low 2D:4D = group with below median ratio, indicating high fetal testosterone. High 2D:4D = group with above median ratio, indicating low fetal testosterone. $^{*}p < 0.05$. Data are displayed as mean ± SEM.

and the BOLD signal were found across both drug groups. There was, however, subthreshold activation in the bilateral parahippocampal cortices that correlated positively with 2D:4D.

3.5.4. *Correlations between testosterone load and BOLD signal for contrast successful > failed navigation across all participants*

Activity in the right hippocampus and parahippocampal cortex, as well in bilateral amygdala was significantly positively correlated with testosterone load (Fig. 2, Table 3).

3.6. *Resting state fMRI*

The resting state fMRI analysis did not reveal any statistical significant differences between the two drug groups.

4. Discussion

In the current study, testosterone administration was found to increase MTL activity related to successful navigation, but did not affect behavioral measures of navigation success even though MRT performance was significantly improved. The results point to increased testosterone levels having a limited effect on spatial cognition, pertaining specifically to performance on tasks probing 3D representations of space, since the testosterone group also performed notably better on the VE direction task. Thus testosterone levels appeared to have direct impact on certain basic aspects of spatial cognition in women, while more complex spatial behaviors, such as navigation, remained unaffected despite adaptive changes in MTL activity indicating improved neuronal processing.

The testosterone group had bilaterally increased MTL activity compared to the placebo group for successful > failed navigation while there was no difference in level of activity for the contrast

Table 3
fMRI results for the contrasts successful > failed navigation, successful > failed navigation with 2D:4D as confounder, and successful > failed navigation correlated with testosterone load.

	MNI–coordinates			Cluster no.	Cluster size	*Z*-score
	X	*Y*	*Z*			
Successful > failed navigation						
Testosterone > Placebo						
Right parahippocampal cortex	15	−32	−13	1	1104	3.68
	18	−23	−18	(1)		3.08
Right amygdala	18	−5	−12	(1)		3.60
	22	−11	−15	(1)		3.16
	28	−7	−10	(1)		3.09
Right hippocampus	30	−20	−14	(1)		2.90
Left parahippocampal cortex	−33	−37	−13	2	1020	3.25
	−26	−45	−6	(2)		3.15
	−21	−38	−12	(2)		3.03
	−10	−45	−5	(2)		3.00
Left amygdala	−10	−11	−11	3	754	3.38
	−15	−6	−14	(3)		3.00
Left hippocampus	−14	−14	−19	(3)		3.23
Placebo > Testosterone						
	ND	ND	ND	ND	ND	ND
Successful > failed navigation with 2D:4D as confounder						
Testosterone > Placebo						
Left hippocampus	−15	−14	−19	1	612	3.37
	−25	−20	−18	(1)		3.10
	−28	−24	−14	(1)		3.07
Left amygdala	−15	−6	−14	(1)		3.22
	−19	−10	−12	(1)		3.16
Right amygdala	18	−5	−12	2	471	3.74
	23	−6	−10	(2)		3.11
Right hippocampus	22	−12	−16	(2)		3.40
	23	−19	−18	(2)		3.30
	23	−17	−16	(2)		3.27
Placebo > Testosterone						
	ND	ND	ND	ND	ND	ND
Successful > failed navigation, correlated with testosterone load						
All participants						
Left amygdala	−15	−7	−15	1	517	3.55
	−20	−11	−11	(1)		3.42
	−15	−6	−13	(1)		3.36
	−12	−9	−16	(1)		3.26
	−15	−8	−17	(1)		3.05
Right parahippocampal cortex	15	−32	−13	2	465	3.77
Right hippocampus	23	−19	−18	(2)		3.69
	22	−12	−16	(2)		3.29
	30	−20	−14	(2)		2.62
Right amygdala	17	−5	−12	3	413	3.44
	19	0	−16	(3)		3.09

The analyses were carried out using the MTL mask and $Z > 2.3$, cluster-corrected at $p < .05$. MNI: Montreal Neurological Institute.

navigation > baseline. This provides empirical evidence for a specific effect of testosterone on neuronal activity related to successful task completion, as opposed to a more generalized increase in neuronal activity shown in previous studies [52–57]. The overall level of activity (*z*-scores) was generally higher throughout the entire MTL in the testosterone group, as reflected in the results of the direct group comparison. The significant correlation between testosterone level and MTL activity during successful navigation, suggested that the genomic effect of testosterone on MTL activity was directly dependent on s-testosterone load. The increased hippocampal activity in the testosterone group during successful navigation was located anteriorly, with coordinates similar to those found when correlating VE navigation activity with success [26] and correlating VE navigation activity with precise knowledge of the global directions within a VE in men [25]. These results suggest that differences in hippocampal activity found during successful navigation in the present study reflect improved spatial representations of the environment considered to reflect allocentric strategies. It should be noted that increased amygdala activity during navigation is uncommon, although observed in some studies in men [25,27]. When examining the two drug groups separately, amygdala activity was only present in the testosterone group, even when examining sub-threshold activation (results not shown). This demonstrated a specific effect of testosterone on amygdala activity. Interestingly, both androgen and estradiol receptors are particularly densely expressed in the adult amygdala [94–96], and the structure is enriched with the testosterone converting enzyme aromatase [97]. Indeed, a common finding across previous studies of testosterone administration in women is that testosterone alters amygdala activity and connectivity; for example, testosterone increases amygdala activity in response to emotional faces [53,55] and reduces the functional coupling between the amygdala and orbitofrontal cortex during face judgment tasks [54,58].

Surprisingly, the specific increase in MTL activity was not accompanied by increased navigation performance in the testosterone group. MTL activity has been shown to increase during successful navigation, is associated with improved performance, and is higher in men compared with women during navigation [25,26,98,99]. It was therefore unexpected that the observed MTL

BOLD signal increase in the testosterone group was accompanied by similar performance as in the placebo group. We speculate that the women were unable to take advantage of the altered neuronal activity and instead continued to rely on an egocentric navigation strategy, as demonstrated by the SSSQ. Even though they had better representations of the direction between landmarks in the VE, they did not to take advantage of this information and make shortcuts. This was demonstrated by the finding that both drug groups used the same amount of time to reach the target landmarks. Lack of experience with 1st person computer games among the participants may have made them less able to take advantage of the testosterone effects during VE navigation. Supporting this notion is the finding of significantly better performance on the VE direction test in the testosterone group, which is less dependent on computer game experience. Adding computer game experience as a covariate in analyses of sex differences in navigation has produced conflicting results, with some studies reporting diminished sex differences [100,101], whereas others find no effect [12]. Furthermore, one dose of testosterone may have positive effects on simpler tasks such as the MRT and VE direction test, and at the same time be insufficient to alter complex learned behaviors such as navigation. It is also important to take into account the difficulty of a task, since ceiling or flooring effects may mask group differences. Indeed, the VE related knowledge measurements were increasingly difficult, as reflected by the participants' mean scores (recognition: 99%, distance: 63%, direction: 47%). As such, it might be that an effect of testosterone is only observed when the task becomes difficult enough to avoid ceiling effects. Indeed, in addition to the large effect seen in the direction test ($d = 1.06$), a non-significant improvement was also seen in the distance test scores ($d = .43$). Alternatively, the distribution of testosterone receptors may be such that regions subserving 3D representations of space (MRT and VE direction) are enriched with testosterone receptors and/or more amendable to its effects. Further, it is also possible that the increased BOLD signal in the testosterone group reflects changes in neuronal excitability per se, i.e., not related to functional or behavioral effects. Neuroactive steroids have been shown to increase neuronal excitability [102], which in turn could result in the increased BOLD signal observed in the current study. It has been suggested that the effects of androgens on hippocampal spine density could involve a rapid GABA-mediated disinhibition of pyramidal cells in the hippocampus [103]. If indeed testosterone works by disinhibiting glutaminergic pyramidal cells in the hippocampus, which increases excitability, this could be a possible biological model for the increase in BOLD response seen in the present study. However, increased neuronal excitability does not by itself predict improved performance. Furthermore, navigation in general should lead to increased BOLD signal in this model, while we observed increased MTL activity only for the contrast successful > failed navigation. Thus a general increase in neuronal excitability seems unlikely although testosterone levels have been shown to correlate with a nonspecific increase in brain activity in women [104]. Taken together, in women testosterone administration had a significant and specific physiological effect on the BOLD signal in the MTL related to successful navigation, which did not translate into improved navigation.

Testosterone did not change our measures of resting state functional connectivity. Small, but robust differences in resting state networks have been demonstrated between men and women [105], and a recent resting state fMRI study showed that the neurosteroids allopregnanolone and dehydroepiandrosterone reduced resting state functional connectivity of the amygdala with frontal, parietal and temporal areas [106]. Moreover, different testosterone derivatives have been shown to change the functional connectivity between amygdala and cortical regions [54,58]. The dual-regression approach that was implemented in the present study has been demonstrated to be the method of choice in detecting group differences in resting state functional connectivity [105]. Previously, seed-based functional connectivity approaches have been used in studies with exogenous testosterone which have shown increased functional coupling of the amygdalae after testosterone administration [54,58]. It should be noted that we did not investigate task-related functional connectivity as in the abovementioned studies, but rather functional networks during rest. Generally, sex differences in resting state networks are weak and require large samples to reach significance [105], which might explain the lack of significant effect of testosterone on resting state networks in this study.

Testosterone administration was associated with improved performance on MRT, in accordance with previous work [10]. The effect size found in the current study ($d = .90$) is comparable to studies of testosterone effects in women ($d = .95$) [10] and to studies comparing men and women (mean $d = 0.94$) [2]. In addition, we found a positive correlation between testosterone load and MRT performance, further supporting the notion that women with relatively high concentrations of testosterone perform better than their counterparts on MRT [107]. The MRT was performed outside the scanner as the focus of this paper was navigation and navigation related MTL activity after testosterone administration, and a review of neuroimaging studies of mental rotation has shown that MRT performance primarily rely on activity in parietal and frontal regions which are outside the FOV used currently [43]. A positive correlation between parietal BOLD response and endogenous testosterone concentration in women during MRT performance has been found, but the effect of successful versus failed trials was not specifically investigated [108]. Thus, the neuronal correlates specifically underlying testosterone's positive effects on MRT remain unknown. MRT performance and VE navigation success tended towards ($p = .053$), but were not significantly correlated, in contrast to what has previously been reported when comparing performance on the MRT and a vMWT [7,8]. The correlation coefficient in the current study and in [7,8] are quite similar though. The lack of a significant correlation might reflect the complexity of the current environment, which stand in contrast to the simpler design of the vMWT. Additionally, the vMWT is a direct test of allocentric navigation whereas tests in the current environment with all its landmarks could be completed equally well relying on egocentric information, which was the preferred strategy in both the testosterone and the placebo groups in this study.

The present study did not detect any significant interaction between 2D:4D ratio, as measure of fetal testosterone exposure, and adult testosterone administration. There was a trend towards a positive effect of fetal testosterone levels on MRT, possibly suggesting a weak organizational effect of testosterone, modulating adult testosterone responsivity on the most testosterone sensitive test. There was also a trend towards a positive correlation between the 2D:4D ratio and parahippocampal activity, and group differences in parahippocampal activity disappeared when including 2D:4D ratio as a confounder. Thus the organizational effects of testosterone appeared to be limited to the parahippocampal cortex. This finding is in line with evidence pointing to a protracted postnatal development of the hippocampus and possibly the amygdala, which may make these structures less affected by the organizational effects of testosterone [109,110]. The limited effect of 2D:4D ratio on MRT performance is divergent from the role of 2D:4D ratio for empathy, which is reported to explain >50% of the variance [23]. The spatial abilities tested here appeared to be either unaffected by exogenous testosterone as well as 2D:4D ratio (i.e., navigation ability and distance test), or largely explained by exogenous testosterone and trending towards an effect of 2D:4D ratio (MRT, direction test and parahippocampal BOLD signal). Taken together, these findings

underscore the complex effects that testosterone has on the female brain from fetal life to adulthood.

4.1. Methodological considerations

The current study included a reliable method for measuring several hormones and hormone binding proteins, and testosterone administration led to an isolated, significant increase in testosterone levels. We observed large variance in the serum concentration of testosterone after 30 min (range 11–97-fold increase from baseline). Similarly, a large variance in clearance rate was observed (range 80–91% reduction between 30 and 100 min). This variability may be due to individual differences in the concentration of other hormones, sex binding proteins, or other factors. With such a large variance, the use of AUC of s-testosterone between our three measurements is a more representative measure of total testosterone load at the individual level than peak serum concentration of testosterone [111]. Inclusion of naturally cycling women who have lower levels of SHBG might have increased the free fraction of testosterone [69], which in turn could potentiate testosterone's effects on spatial abilities. Still, the testosterone dose vastly exceeded the binding capacity of SHBG in the non-naturally cycling women included. We therefore do not believe that using non-naturally cycling women impacted on the results. It should be noted that previous studies that included women on oral contraceptives report significant effects of oral testosterone on behavior [23,52,54]. Including women that were not matched on menstrual phase would have introduced numerous biases caused by fluctuating and varying hormone levels [9,67,68]. By including women during the phase when sex hormones are at the lowest level, we opted to maximize any effect of exogenous testosterone in this first study of testosterone administration effects on female navigation. This approach does, however, reduce the generalizability of our results. Moreover, the time interval between testosterone administration and primary task performance outcome was four hours in the current study, similar to that in previous studies. Still, no effect of testosterone was detected on navigation behavior in the current study although the MRT performance effect was as previously described in the literature. Whether testosterone administration affects all higher cognitive functions with a similar time course is unknown. However, using a four-hour time window has previously been shown to affect a variety of measures [10,23,52,54,60]. The current study also found significantly improved MRT and direction test performance as well as increased BOLD signal around four hours after testosterone administration, showing that other effects than choice of time window are likely to underlie the lack of effect of testosterone on navigation performance.

It should also be noted that the restricted FOV during fMRI and the use of an anatomical mask in the analysis of the fMRI data made areas outside the MTL unavailable for analysis. Thus, differences in activity in other brain areas such as the retrosplenial cortex, precuneus or caudate nucleus, which might be expected based on the participants' inclination towards an egocentric navigation strategy, could not be addressed. The focus of the current study was, however, on differences in the MTL since direct evidence of activational effects of testosterone is largely limited to this area [36]. In addition, sex differences in virtual navigation may have been easier to detect in VEs with only distal reference points [112]. The VE in the current study consisted of both distal and proximal cues in order to mimic an ecologically valid navigation situation with both proximal and distal landmarks. A surprisingly large effect of testosterone administration was found on nausea. However, no correlation was found between performance in the VE and level of nausea, and we therefore do not believe this difference affected our results. Finally, a note should be made regarding the statistical thresholds used. In this study, we have grouped the variables into four "families" that have been treated separately: the baseline comparisons, the hormonal measurements, the fMRI results and the behavioral outcome measurements. Tests within a family were corrected for multiple comparisons using the Benjamini-Hochberg method [89] which has been shown to work even with dependent measurements [113] such as for several of the behavioral outcome measures. No corrections for multiple comparisons were applied for the correlation analyses. Caution should therefore be made when interpreting these findings.

5. Conclusions

The current study confirmed that there is a relationship between testosterone load and mental rotation abilities in healthy women. We further showed that women who received testosterone had significantly improved representation of the direction within the VE. No effects of testosterone on navigation performance were found despite increased brain activity within the MTL specifically during successful navigation in the testosterone group. In the MTL, navigation activity in the parahippocampal cortex was shown to be sensitive to both activational and organizational testosterone effects. Why certain behaviors and certain brain regions were more sensitive to testosterone remain uncertain. We speculate that this is either related to differences in the effect of exogenous testosterone in brain regions supporting the task at hand (localization and concentration of receptors, enzyme activity, and organizational effects), and/or that some tasks can be solved equally well using different strategies and are therefore less susceptible to the effects of testosterone, and more dependent on habit.

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