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Original Article

ORIGINAL ARTICLE

Effects of Adult Female Rat Androgenization on Brain Morphology and Metabolomic Profile

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Abstract

Androgenization in adult natal women, as in transsexual men (TM), affects brain cortical thickness and the volume of subcortical structures. In order to understand the mechanism underlying these changes we have developed an adult female rat model of androgenization. Magnetic resonance imaging and spectroscopy were used to monitor brain volume changes, white matter microstructure and ex vivo metabolic profiles over 32 days in androgenized and control subjects. Supraphysiological doses of testosterone prevents aging decrease of fractional anisotropy values, decreased general cortical volume and the relative concentrations of glutamine (Gln) and *myo*-Inositol (mI). An increase in the *N*-acetylaspartate (NAA)/mI ratio was detected d. Since mI and Gln are astrocyte markers and osmolytes, we suspect that the anabolic effects of testosterone change astrocyte osmolarity so as to extrude Mi and Gln from these cells in order to maintain osmotic homeostasis. This mechanism could explain the brain changes observed in TM and other individuals receiving androgenic anabolic steroids.

Key words: anabolic androgenic steroids, female androgenization, female-to-male transsexuals, MRI, transsexual men

Introduction

Testosterone is known for its androgenic and anabolic effects. Anabolic-androgenic steroids (AAS) are synthetic substances derived from testosterone used for their masculinizing and/or trophic effects on the body. Because of these effects, AAS are administered to hypogonadal men as replacement therapy (Borst and Yarrow 2015) and to female-to-male transsexuals (transsexual men [TM]) for virilization (Gooren 2011). Bodybuilders and weightlifters consume these substances. In addition, testosterone has been prescribed to treat fatigue, erectile dysfunction, and psychosexual dysfunction (Aversa et al. 2004; Tsujimura 2013; Gannon and Walsh 2016). Although the hormone might be neuroprotective (Melcangi et al. 2016), chronic administration of AAS is associated with adverse

effects (Pomara et al. 2015) in addition to cognitive deficit (Kanayama et al. 2013; Kaufman et al. 2015) and psychiatric disorders in men and women (Pope and Katz 1988; Perry et al. 1990; Gruber and Pope 2000; Ip et al. 2010). Moreover, it has been suggested that nonmedical use of AAS could have a neurodegenerative potential (Pomara et al. 2015).

Despite the literature on adverse AAS effects, little attention has been paid to their effects on the brain. Bjørnebekk et al. (2016) compared the effects of long-term (more than 1 year) AAS and non-AAS in weightlifters and found decreases in the volumes of total gray matter, cerebral cortex, and putamen as well as a thinner cortex in AAS consumers. They also reported a negative correlation between AAS use and brain volume and cortical thickness. The same group also reported reduced functional connectivity between the amygdala and default-mode network and between the dorsal attention network and frontal node (Westlye et al. 2016). Although the response may inherently differ irrespectively of time in comparison to cis persons, medium term (6 months to 1 year) administration of AAS to masculinize TM increases their total brain volume (Hulshoff Pol et al. 2006) and cortical thickness bilaterally in the postcentral gyrus and unilaterally in the left hemisphere inferior parietal, lingual, pericalcarine, and supramarginal areas as well as the right hemisphere rostral middle frontal and the cuneus, in a manner that positively correlates with serum testosterone levels in parieto-temporo-occipital regions (Zubiaurre-Elorza et al. 2014). There are also increases in the volume of the hypothalamus (Hulshoff Pol et al. 2006) and the right thalamus (Zubiaurre-Elorza et al. 2014). Fractional anisotropy (FA) increases were also reported in the right superior longitudinal tract and the right corticospinal tract (Rametti et al. 2012). The androgenization of TM is a unique situation of androgenizing biological females. Influential clinical reviews suggest that the cross-sex hormone treatment is reasonably safe (Moore et al. 2003; Gooren et al. 2008; Wierckx et al. 2012).

Testosterone and its metabolites affect cell functions in the brain. Originated from the peripheral steroidogenic glands (testes, ovaries, adrenals), testosterone is also produced by neurons and glial cells (Baulieu 1998) and acts through complex and multiple routes in the brain. It uses genomic and nongenomic pathways that are probably interlinked; mammals, including humans, express androgen (AR) and estrogen (ER) receptors (Simerly et al. 1990; Finley and Kritzer 1999; Osterlund et al. 2000; Lorenz et al. 2005). In rodents (Baulieu 1998) and humans (Stoffel-Wagner et al. 1998; Steckelbroeck et al. 1999), testosterone is converted to estradiol in the brain via the p450 enzyme aromatase, which shows sex differences (Tabatadze et al. 2014), and ultimately acts on the ER. Testosterone is also reduced to dihydrotestosterone, which acts directly via the AR. However, other reduced metabolites (3α diol and 3β diol) also bind to ER. What is more, neurons and glial cells of rodents and humans have the molecular machinery for steroidogenesis (Melcangi et al. 2016).

Magnetic resonance imaging (MRI) and spectroscopy have shown enormous potential for research into the mechanisms of brain function under physiological or pathological conditions (Liu 2015). Implementing an animal model for adult female

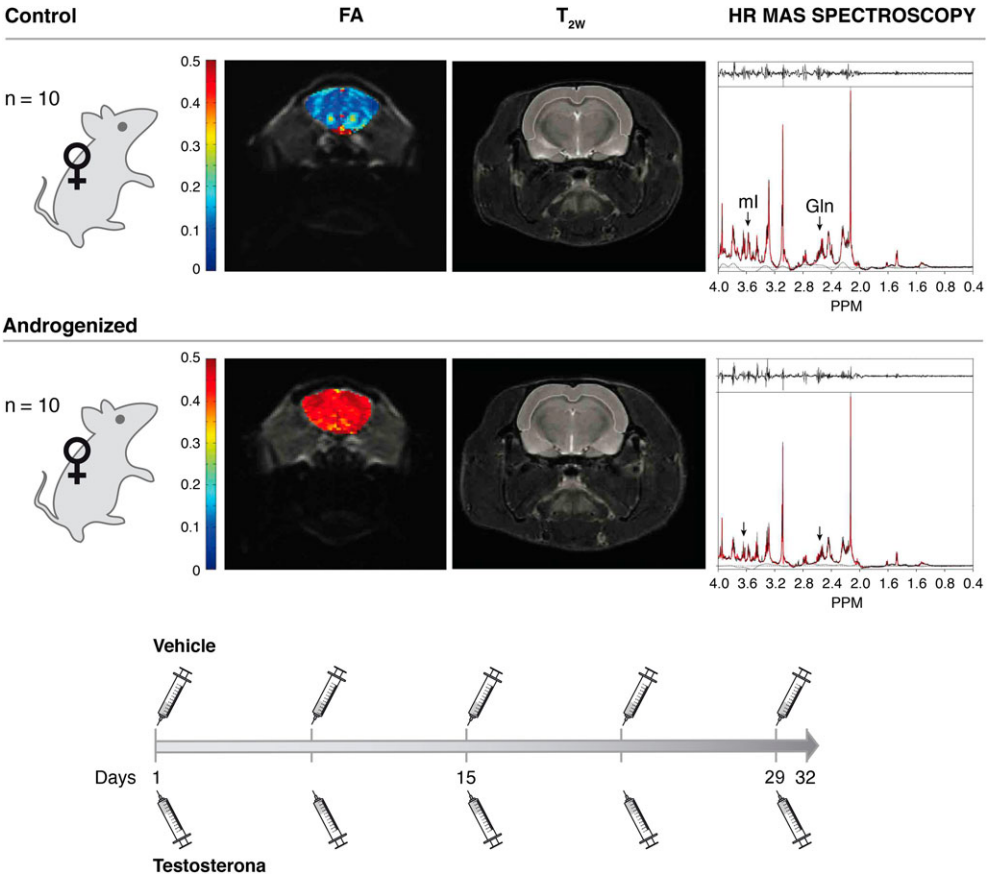

Figure 1. Experimental design. Adult female rats received either vehicle (upper panels) or testosterone (lower panels), at the indicated times. Testosterone levels were determined by radioimmunoassay (RIA) on Days 1, 15, and 32. T_{2w} measurements of cortical volume and cerebral fractional anisotropy (FA) values were measured in vivo by MRI on the same days. Metabolomic profiles were obtained in brain biopsies of dissected regions by ^{1}H HRMAS on Day 32, after cerebral microwave fixation.

androgenization would be an important tool to better interpret the results gathered in humans and to develop advanced therapeutic strategies. Increases in FA (Rametti et al. 2012), cortical, and subcortical volume (Hulshoff Pol et al. 2006; Zubiaurre-Elorza et al. 2014) and cortical thickness (Zubiaurre-Elorza et al. 2014) after short-medium term treatment with testosterone in TM have suggested the anabolic effects of this hormone could lead to an increase of molecules that can attract water to enter brain cells.

In the present work, we test this hypothesis in a longitudinal study of androgenized female rats that lasted 32 days, measuring their cortical volume and FA by mean of MRI acquisition and diffusion tensor imaging (DTI) respectively; ex vivo brain tissues were analyzed using proton magnetic resonance spectroscopy (^{1}H-MRS) to assess the metabolic profile of neurochemical metabolites.

Materials and Methods

Subjects

Twenty 80-day-old female Wistar rats (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid Spain) were randomly divided into 2 groups: control (n = 10) and experimental (n = 10). Initial weight of the animals was 204.33 ± 3.94 g (control) and 201.9 ± 3.79 g (experimental). They were housed 5 animals per cage and kept in a room at a constant temperature of 22 ± 2°C, humidity 40% and lights on from 8:00 to 20:00, with food and water ad libitum. One control subject died on Day 22. Data from this subject were not included in statistical analyses.

General Description of the Design

The experiment lasted 32 days (Fig. 1). On the first day (D1) blood samples for testosterone radioimmunoassay (RIA) were taken from the tails of the animals before they were anesthetized and placed in the scanner chamber for brain MRI acquisition to obtain T2 weighted spin-echo images and DTI. After the acquisition procedure (Days 1, 15), experimental subjects were injected with 40 mg/kg of testosterone propionate (TP) (Sigma Aldrich, Madrid, Spain) (Callies et al. 2003), while control subjects were injected with the vehicle (olive oil). On Days 7, 22, and 29 experimental and control subjects were also TP- or vehicle-injected at the same time (8.00–12.00) as on acquisition days.

To maintain a constant dose for each animal's weight in the experimental group, as well as the volume of vehicle for the control group, the volume injected was calculated each time using the following formula:

$$V = \frac{(\text{body weight in grams}/1000) \times \text{dose}}{\text{Testosterone concentration}}$$

Blood Sampling and Hormonal Assay

Blood samples were obtained under anesthesia from the tail vein into precooled vials on Days 1, 15, and 32 before MRI acquisition. Samples were centrifuged at 2000 rpm for 5 min at 4°C and plasma stored in marked precooled labeled vials at –20°C until assay.

Plasma testosterone concentrations were measured by RIA using a commercial kit distributed by DRG (DRG International Inc., Springfield, NJ 07081, USA), CV 4.6. Briefly, a fixed amount of I-125 labeled testosterone competes with the testosterone to be measured present in the sample or in the calibrator for a fixed amount of antibody sites being immobilized to the wall of a polystyrene tube. Neither extraction nor chromatography is required because of the high specificity of the coated antibodies.

After 3 h' incubation at 37°C, an aspiration step terminates the competition reaction. The tubes are then washed with 3 mL of wash solution and aspirated again. A calibration curve is plotted and the testosterone concentrations of the samples are determined by dose interpolation from the calibration curve.

The specificity of the antibody as the percentage of cross-reaction as estimated by comparison of the concentration yielding a 50% inhibition was: <0.31% for dihydrotestosterone; <0.28% for androstenedione and <0.004% for other compounds. The intra- and interassay coefficients of variation as measurements of intra- and interassay precision were 4% and 5.1%, respectively, and the sensitivity or detection limit was 0.05 ng/ml.

Magnetic Resonance Imaging

MRI acquisitions were performed on a Bruker Pharmascan system (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0-T horizontal-bore superconducting magnet, equipped with a quadrature 40 mm coil and a 90 mm Bruker gradient coil insert (maximum intensity 360 G/cm). All data were acquired using a Hewlett-Packard console running Paravision 5.1 software (Bruker Medical Gmbh) operating on a Linux platform.

The Wistar rats were placed into the center of the volume radiofrequency (RF) coil and positioned in the magnet under continuous inhalation anesthesia via a nose cone. A respiratory sensor connected to a monitoring system (SA Instruments, Stony Brook, NY) was placed under the abdomen to monitor the rate and depth of respiration. The subjects were anesthetized with 2% isofluorane in 1 L of oxygen in an induction chamber and the flow of anesthetic gas was constantly regulated to maintain a breathing rate of 50 ± 20 bpm. Body temperature was maintained at approximately 37°C by passing warm water through a heat exchanger machine into the animal platform. The physiological state of the rats was monitored using an MRI compatible small animal gating system from SA Instruments (Stony Brook, NY, http://www.i4sa.com) that registered the respiratory rate.

T_2-weighted ($T_{2\text{-W}}$) spin-echo images were acquired with a rapid acquisition relaxation enhancement (RARE) sequence in axial orientations using the following parameters: TR = 3000 ms, TE = 44.3 ms; RARE factor = 8; Av = 3; FOV = 3.5 cm; acquisition matrix = 256 × 256 corresponding to an in-plane resolution of 148 × 148 μm^2, slice thickness = 1.50 mm without gap and number of slices = 16.

Diffusion weighted images used the following acquisition conditions: TR = 4000 ms; TE = 40 ms; Av = 1; diffusion gradient duration = 3.5 ms, diffusion gradient separation = 20 ms, 7 gradient directions; acquisition matrix = 128 × 128 corresponding to an in-plane resolution of 312 × 312 μm^2 and 2 b values (100 and 1400 s/mm^2).

ADC maps were obtained by a linear fitting of the logarithm of signal intensity (S) versus the b factor according to the expression: $S_b = S_0 \exp(-\text{ADC} \times b)$, using the homemade software MyMap Analyzer, based on MatLab v7.0 scripts. ADC value was extracted from maps using the region of interest (ROI) with Image J software.

Sacrifice and Dissection

On Day 32, immediately after the MRI session, rats were sacrificed by focused microwave irradiation, with a Muromachi

Microwave 5KW Model TMW-6402C (Toshiba, Tokyo, Japan) to obtain a rapid heat inactivation of brain enzymes. Rats were anesthetized previously and placed in a water-jacketed holder prior to insertion into the system. A power setting of 5 kW and an exposure time of 2.7 s was used. Following sacrifice, the skull was quickly opened and brain dissected to obtain the following regions: olfactory bulb-frontal pole; fronto-parietal region of the right hemisphere and the hippocampus from the left hemisphere (Paxinos and Watson 1996).

Ex Vivo ^{1}H HRMAS

The ex vivo ^{1}H spectra were performed on a Bruker Avance 11.7 T spectrometer (Bruker Biospin, Karlsruhe, Germany) equipped with a 4 mm triple channel ^{1}H/^{13}C/^{31}P HR-MAS (High Resolution Magic Angle Spinning) probe operating at 4°C. Samples (10–15 mg) were introduced into a 50 µl zirconium oxide rotor (4 mm OD) with 50 µl D_2O and cylindrical insert and spun at 5000 Hz at 4°C to decrease tissue degradation processes. Two types of mono-dimensional proton spectra were acquired using a water suppressed spin echo Carr Purcell Meiboom Gill (CPMG) sequence with 36 ms and 144 ms echo time and 128 scans; data were collected into a 64 kH data point using a spectral width of 10 kHz (20 ppm), water presaturation during relaxation delay of 2 s and total acquisition time of approximately 19 min. All spectra were processed using LC Model version 6.2-OR (Stephen Provencher, Oakville, ON, Canada), a prior knowledge spectral fit software. Only metabolites fitting within a Cranmer-Rao Lower Bound of 20% were considered.

Estimation of Neocortex Volume

Neocortex volume was bilaterally estimated using the online ImageJ package for Windows, version 1.48 v program (https://www.google.es/?gws_rd=ssl#q=ImageJ+-+Java, National Institute of Health, Bethesda, USA). We made 3 series of measurements that were taken from the RM slides obtained on Days 1, 15, and 32 of the experiment. Contiguous MRI slices were ordered sequentially in the rostral-caudal direction and the neocortical perimeter was delineated in every image by comparison with the corresponding plates of a brain atlas (Paxinos and Watson 1996). Images limiting the neocortical volume measurements were the rhinal fissure and the midline (Fig. 1). Neocortical volume was calculated through the algorithm: $V = \sum a \times h$, in which a is the measured area analyzed in every RM slice and h the distance between slices (1500 µ). As ImageJ automatically provides the value of the delineated area in mm^2, we multiplied the sum of the estimated areas by 1.5 to obtain the total volume of the neocortex in mm^3.

Statistical Analyses with SPSS

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). Pairs of within-subjects ANOVA analyses with experimental condition (control vs. treatment) and day of treatment (D1 and D15 vs. D15 and D32) effect on testosterone levels, total cortical volume (TCV), and right and left hemisphere volume were run. A pair of hierarchical regression analyses controlling for the TCV on D1 and including the increase in testosterone between D1 and D15, and between D15 and D32 as predictors of the cortical volume on D32 were conducted for the experimental and control groups, respectively. A within-subject ANOVA analysis with experimental condition (control vs. treatment) and day of treatment (D1 and D32) on white matter microstructure was conducted. Hierarchical regression analyses were used to identify the effect of the increase in testosterone between D1 and D32 on FA values on D32 controlling for FA values on D1. Analyses of variance, ANOVA, were conducted with experimental condition (control vs. treatment) on the level of Gln, mI, mI + Gly the NAA/mI ratio, and the NAA/creatine ratio. A series of hierarchical linear regression analyses including the effect of the increase in testosterone between D1 and D15, and between D15 and D32 on mI, mI + Gly, and Gln were conducted. The level of significance for all these analyses was set arbitrarily at $P < 0.05$.

Results

Testosterone

A pair of repeated measures analyses on testosterone levels including the experimental condition (control vs. treatment) and day (D) of treatment was conducted. Results showed that testosterone increased in the treated group while no change was detected in the control group. A first repeated measures analysis including experimental condition and day of treatment (D1 vs. D15) yielded a significant 2-way interaction: $F(1,17) = 17.51$, $P < 0.001$, $\eta^2 = 0.51$. Inspection of means indicated that testosterone did not increase in the control condition between D1 and D15, $M = 0.14 \pm 0.09$ ng/mL versus $M = 0.16 \pm 0.85$ ng/mL, $P = 0.98$, $\eta^2 = 0.00$. In contrast, the increase in testosterone in the treatment condition between D1 and D15 was significant, $M = 0.76 \pm 0.08$ ng/mL versus $M = 5.64 \pm 0.80$ ng/mL, $P < 0.001$, $\eta^2 = 0.69$. A second repeated measures analysis including experimental condition and day of treatment (D15 vs. D32) also yielded a significant 2-way interaction, $F(1,17) = 11.56$, $P = 0.003$, $\eta^2 = 0.40$. Inspection of means indicated that testosterone had not increased in the control condition at D32, $M = 12.11 \pm 1.07$ ng/mL, $P = 0.98$, $\eta^2 = 0.00$. In contrast, the increase in testosterone in the treatment condition at D32 was significant, $M = 5.64 \pm 0.80$ ng/mL, versus $M = 12.11 \pm 1.02$ ng/mL, $P < 0.001$, $\eta^2 = 0.59$.

Cortical Volume

Total Cortical Volume

A pair of repeated measures analyses including experimental condition (control vs. treatment) and day of treatment was conducted on TCV (Fig. 1). A first repeated measures analysis including experimental condition and day of treatment (D1 vs. D15) yielded a significant interaction, $P(1,17) = 4.84$, $P = 0.042$, $\eta^2 = 0.22$. Inspection of means indicated that TCV did not change in the control condition between D1 and D15, $M = 430.51 \pm 5.57$ versus $M = 432.38 \pm 6.82$, $P = 0.63$, $\eta^2 = 0.01$, while there was a significant reduction in the females treated with testosterone, $M = 428.99 \pm 5.28$ versus $M = 419.24 \pm 6.47$, $P = 0.016$, $\eta^2 = 0.30$ (Fig. 2). A second repeated measures analysis including experimental condition and day of treatment did not show a significant interaction effect between D15 and D32, $P = 0.40$, $P = 0.40$.

A hierarchical regression analysis controlling for the TCV in the experimental group on D1, and including the increase in testosterone between D1 and D15, and between D15 and D32 as predictors of the cortical volume on D32 showed that the increase in testosterone between D1 and D15 had a significant effect on the reduction of cortical volume ($b = 0.65$, $t = 2.39$, $P = 0.05$), and the increase in testosterone between D15 and D32 also had a significant effect on the reduction of cortical volume ($b = 1.27$, $t = 3.19$, $P = 0.019$). The effect of the cortical volume at D1 on D32 cortical volume was not significant, $P = 0.17$, which

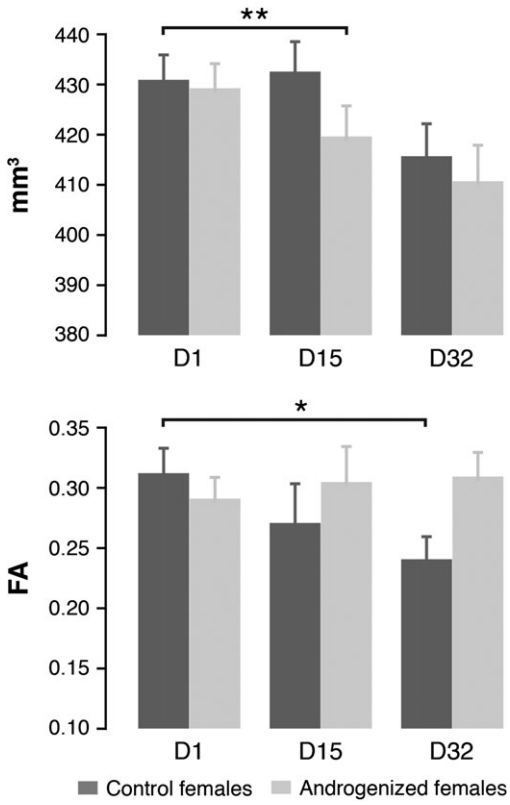

Figure 2. Total cortical volume (mm^3) and fractional anisotropy (FA) values in control (dark) and androgenized (grey) females. Results are shown as mean ± standard deviation of each animal group. *P < 0.02, **P < 0.01.

confirms that changes in the cortical volume of the experimental group are due exclusively to testosterone treatment. The same regression analyses in the control group did not find significant effects of testosterone levels on the D32 cortical volume, Ps > 0.13. The effect of the cortical volume at D1 on the cortical volume at D32 was significant for the control group, b = 0.71, t = 3.36, P = 0.02, indicating that the final cortical volume only depends on the initial cortical volume in that group.

Right and Left Hemisphere Volumes

A similar series of analyses were run separately for the 2 hemispheres. They did not reveal any significant effect, indicating that the reduction in volume was general through the entire cortex.

White Matter Microstructure

Differences in FA between experimental and control groups only reached significance in the region of the olfactory bulb and the pole of the frontal cortex (Fig. 1). A repeated measure analysis including experimental condition (control vs. treatment) on FA on D1 versus D32 showed a significant interaction: $F(1,17) = 5.75$, $P = 0.028$, $\eta^2 = 0.25$. Inspection of means indicated that FA in the olfactory bulb and the frontal pole did not change in the experimental condition between D1 and D32, M = 0.29, ± 0.02 versus M = 0.31 ± 0.02, $P = 0.45$, $\eta^2 = 0.03$, while there was a significant reduction in the control group, M = 0.31, ± 0.02 versus M = 0.24 ± 0.02, $P = 0.02$, $\eta^2 = 0.28$ (Fig. 2). When the analyses were conducted separately for D1 versus D15 and D15 versus D32 the change was not significant.

A hierarchical regression analysis of the effect of the increment in testosterone between D1 and D32 on FA values on D32, controlling for FA values on D1, showed a significant effect of the increment in testosterone on FA values in the olfactory bulb and pole of the frontal cortex on D32, $b = 0.46$, $t(16) = 2.03$, $P = 0.05$, indicating that testosterone treatment prevented the decrease in FA values observed in the control group. Summarizing, testosterone treatment between D1 and D32 maintained FA levels in androgenized females.

Table 1 Metabolite concentrations[a]

	Control		Androgenized	
	Mean	SEM	Mean	SEM
mI	0.82*	0.04	0.68	0.04
mI + Gly	0.80*	0.04	0.65	0.04
Gln	0.74*	0.02	0.67	0.02

mI, *myo*-Inositol; Gly, glycine; Gln, glutamine; *P < 0.05.
[a]Relative concentration to PCh + Cr.

Ex Vivo Metabolic Profiles

In the fronto-parietal cortex, univariate analyses indicated that the level of Gln, mI and mI + Gly were lower in the treatment than in the control condition, $F(1,17) = 5.05$, $P = 0.038$, $\eta^2 = 0.23F(1,17) = 6.69$, and $F(1,17) = 7.29$, respectively, $P = 0.015$, $\eta^2 = 0.30$ (Fig. 1 and Table 1).

A series of additional linear regression analyses including the effect of the increase in testosterone between D1 and D15, and between D15 and D32 on mI, mI + Gly, and Gln indicated that in the 3 cases, the increase in testosterone between D1 and D15 was responsible for the decreases in mI, mI + Gly, and Gln (Table 2).

In order to investigate the regulation of volume in the fronto-parietal cortex an ANOVA analysis was run between experimental and control groups with respect to the NAA/mI ratio, indicating higher levels in the experimental group ($F[1,17] = 6.609$, $P = 0.020$ [control: 1.389 ± 0.081; treated: 1.677 ± 0.0877]). A linear regression analyses including the effect of the increase in testosterone between D1 and D15, and between D15 and D32 on the NAA/mI ratio showed that the increase in testosterone between D1 and D15 was responsible for the increase of the ratio, $b = 0.53$, $t(16) = 2.53$, $P = 0.022$.

We conducted a series of ANOVAs comparing the control and the treatment condition in so far as the glutamate/glutamine, aspartate/glutamine and GABA/glutamine ratios. None of the effects was significant, Ps > 0.21, with the exception of the aspartate/glutamine ratio in the hippocampus, where the difference was marginal ($F[1,15] = 3.64$, $P = 0.076$, $\eta^2 = 0.195$). In addition, the hippocampus of androgenized females showed an almost significant decrease in the GABA/aspartate ratio ($F[1,15] = 4.058$, $P < 0.06$; $\eta^2 = 0.213$).

Discussion

We monitored brain volume changes, white matter microstructure and ex vivo metabolic profiles in androgenized adult female rats to investigate the mechanisms underlying TM cerebral androgenization (Hulshoff Pol et al. 2006; Rametti et al. 2012; Zubiaurre-Elorza et al. 2014). In adult female rats we observed that testosterone: (1) produced a generalized decrease in cortical volume while changes seen in the female controls were due to other causes; (2) prevented the age-related

Table 2 Linear regression of changes in testosterone between Day 1 → Day 15 and Day 15 → Day 32 on mI, mI + Gly, and Gln

	Period	β	t	P
mI	D1–D15	−0.547	−2.657	0.017
	D15–D32	−0.123	−0.595	0.560
mIGly	D1–D15	−0.502	−2.368	0.031
	D15–D32	−0.150	−0.708	0.489
Gln	D1–D15	−0.608	−3.112	0.007
	D15–D32	−0.112	−0.574	0.547

mI, myo-Inositol; Gly, glycine; Gln, glutamine.

decrease in FA values seen in the female control group in the olfactory bulb-frontal pole region; (3) decreased the relative concentrations of Gln, mI, and mI + Gly and the changes in these metabolites were produced in the first 15 days; and (4) produced an increase in the NAA/mI ratio.

Cortical Volume

During brain maturation in rats, the largest cortical volume is achieved at 2 months of age, declining thereafter (Mengler et al. 2014). Thus, our subjects can be considered adult females at the beginning of the experimental period. Notably, an accelerated decrease was detected due to testosterone treatment in comparison to the control females whose cortical volume decreases may be due to ageing only. Under physiological and pathological conditions, testosterone is known to decrease cortical volume and thickness and the volume of subcortical structures in humans (Merke et al. 2003; Raznahan et al. 2010; Bramen et al. 2011; Peper et al. 2011). Long-term AAS consumers show a decrement in cortical and subcortical structure volumes (Bjørnebekk et al. 2016) although a larger right amygdale and reduced connectivity have also been reported (Kaufman et al. 2015). These results contrast with those of Hulshoff Pol et al. (2006) reporting an increase in cortical and subcortical volumes, and those by Zubiaurre-Elorza et al. (2014) showing increased subcortical volumes and cortical thickness after short-medium term TM androgenization. It seems that exposure time to AAS is an important variable. In the present study, we show that the decrease in cortical volume of the adult female rat brain, is due to testosterone administration.

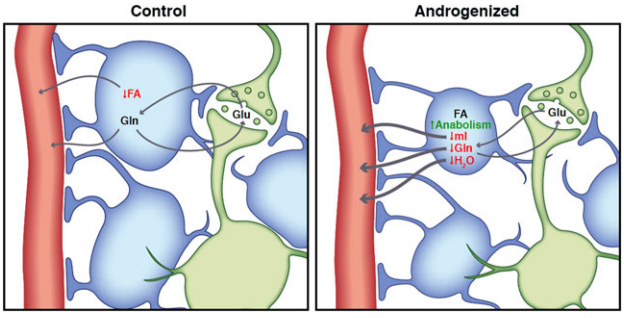

Figure 3. Effects of adult female androgenization on cerebral neuroglial interactions. Adult female androgenization results in decreases in glutamine (Gln) and *myo*-inositol (mI), 2 osmolytes and astrocyte biomarkers. Testosterone triggers increased protein synthesis, cortical volume reductions and relative FA increases, paralleled by osmotically driven reductions in levels of Gln and mI levels, which are extruded to the extracellular space and capillaries, thus decreasing astrocytic volume. Gln extrusion may decrease intra-astrocytic Gln concentrations, altering flux through the glutamate–glutamine cycle.

Fractional Anisotropy

We observed a step-by-step decrease in FA in the control group over the course of the experiment, most probably due to the ageing effect, as seen in humans (Bennet et al. 2010; Salami et al. 2012). However, we found no changes in FA values in androgenized females over the same experimental period, suggesting that the testosterone treatment prevented age-dependent FA decreases. This supports our previous interpretation that the anabolic effect of testosterone would explain FA increases in TM (Zubiaurre-Elorza et al. 2014).

Metabolite Profile

The cerebral metabolic profiles of androgenized females showed a decrease in the relative concentrations of mI and Gln in the fronto-parietal region. These decreases were negatively correlated with the increases in testosterone levels observed between D1 and D32. Since mI and Gln behave as major osmolytes, these changes suggest that testosterone levels may alter volume regulation processes, and this may underlie the changes in cortical volume observed above.

A major organic osmolyte, mI regulates cell volume in response to osmotic stress (Yancey et al. 1982). It is also an astrocyte marker and the precursor of the phosphatidylinositol second messenger system (Brand et al. 1993). The mI decreases we observed were negatively correlated with testosterone levels associated with a decrease in cortical volume. Ionic and osmolyte extrusion drive intracellular water efflux, which is what produces the decreased cortical volume associated with testosterone. The anabolic effects of testosterone increased molecular synthesis in astrocytes, imposing increased water uptake with the concomitant osmotic volume increase. To maintain constant cerebral volume and prevent astrocytes from swelling during testosterone treatment, we propose that mI and Gln would be extruded from the astrocytes to the extracellular space and subsequently eliminated through the cerebrospinal fluid, thus osmotically decreasing water content associated to the anabolic testosterone effects. This proposal coincides with the observation that mI and Gln contents are depleted in androgenized female rats. This mechanism could also explain the quick decrease observed in cortical volume in androgenized compared with control females. Astrocytes are the only type of brain cells capable of Gln synthesis (Martínez-Hernández et al. 1977). Gln is the precursor of excitatory (glutamate, aspartate) and inhibitory (γ-amino butyric acid) amino acids. Gln decreases could be related to its function as an osmolyte as discussed above. In addition we found signals that the equilibrium of the excitatory system was distorted in androgenized adult female rats because the GABA/aspartate and aspartate/glutamine ratios were decreased.

General Comment

Our results are much better understood from the vantage point of neuron–astrocyte relationships (Magistretti and Pellerin 1996) (Fig. 3). Gln and mI, 2 astrocyte markers, are concurrently affected by androgenization in adult female rats. Astrocytes have the ability to change their volume to maintain their osmolarity (Zwingman and Butterword 2005). Androgenized female rats showed depletion of Gln and mI in the olfactory bulbs and the frontal pole. These concentration changes suggest a response to the anabolic effects of testosterone as revealed by the maintenance of the FA values in androgenized females in spite of ageing. In addition, Gln depletion suggests that

neuronal–astrocyte interactions might also be affected in the glutamine–glutamate cycle essential for excitatory neurotransmission, as supported by a decrease in the GABA/aspartate and aspartate/glutamine ratios. Testosterone can exert either protective or damage-promoting effects in primary cortical astrocytes (Gatson and Singh 2007), and we did not find changes in the relative NAA concentration, considered to be a neuronal marker (Block et al. 2002). However, our results suggest that the observed changes represent an essential aspect that merits routine screening in TM and women receiving AAS.

The hormone we measured was testosterone and we have proved that all changes found were due to this hormone. However, we do not know how steroidogenesis in neurons and astrocytes is affected by high doses of AAS. Almost all testosterone metabolites show a positive correlation between plasma and cerebral cortex (Caruso et al. 2013) and it was demonstrated that the cortex adapts its local levels of neurosteroids in response to changes of gonadal hormones in a region-specific manner (Caruso et al. 2010).

Comparison with Humans

Long-term use of AAS by weightlifters decreases the volume and thickness of the cerebral cortex (Bjørnebekk et al. 2016) while medium term administration of AAS to masculinize TM increases FA values (Rametti et al. 2012) as well as cortical volume (Hulshoff Pol et al. 2006) and thickness (Zubiaurre-Elorza et al. 2014). Overall, our model seems to fit more with long-term administration of AAS.

It could be argued that in rodents the estradiol obtained from the aromatization of testosterone is the hormone that plays the main role in the metabolic effects of testosterone while in humans the hormone may be testosterone and its reduced metabolites the main actors. It should be remembered that the brain of humans has the molecular machinery for testosterone aromatization and reduction and that reduced DHT metabolites bind the ERs. This informs our female rat model.

Strengths and Limitations

To the best of our knowledge, this is the first experimental study on the effects of supraphysiological doses of testosterone in female rats. The main strength of our study is its longitudinal design, which makes it possible to causally relate changes in cortical volume, white matter microstructure and brain metabolites to serum testosterone levels. However, we envision some limitations. The first is experiment duration. Perhaps a longer exposure to testosterone would allow us to discern if chronic testosterone administration would be able to perturb neuron–astrocyte interactions in an irreversible manner. Second, it could be argued that we did not test a possible dose effect. However, we only intended to characterize the possible effects of a supraphysiological dose of testosterone, in a rat female model, to explain previous findings in TM and women consuming AAS.

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Notes

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