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Effects of adult male rat feminization treatments on brain morphology and metabolomic profile

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ABSTRACT

Body feminization, as part of gender affirmation process of transgender women, decreases the volume of their cortical and subcortical brain structures. In this work, we implement a rat model of adult male feminization which reproduces the results in the human brain and allows for the longitudinal investigation of the underlying structural and metabolic determinants in the brain of adult male rats undergoing feminization treatments. Structural MRI and Diffusion Tensor Imaging (DTI) were used to non-invasively monitor in vivo cortical brain volume and white matter microstructure over 30 days in adult male rats receiving estradiol (E_2), estradiol plus cyproterone acetate (CA), an androgen receptor blocker and antigonadotropic agent (E_2 + CA), or vehicle (control). Ex vivo cerebral metabolic profiles were assessed by ^{1}H High Resolution Magic Angle Spinning NMR (^{1}H HRMAS) at the end of the treatments in samples from brain regions dissected after focused microwave fixation (5 kW). We found that; a) Groups receiving E_2 and E_2 + CA showed a generalized bilateral decrease in cortical volume; b) the E_2 + CA and, to a lesser extent, the E_2 groups maintained fractional anisotropy values over the experiment while these values decreased in the control group; c) E_2 treatment produced increases in the relative concentration of brain metabolites, including glutamate and glutamine and d) the glutamine relative concentration and fractional anisotropy were negatively correlated with total cortical volume. These results reveal, for the first time to our knowledge, that the volumetric decreases observed in trans women under cross-sex hormone treatment can be reproduced in a rat model. Estrogens are more potent drivers of brain changes in male rats than anti-androgen treatment.

1. Introduction

Transgender women often experience lifelong gender dysphoria due to a gender assignment at birth that is incongruent with their gender identity. They frequently seek hormone therapy. High doses of estrogens, coupled with an antiandrogen, are pharmacologically used to feminize the body for gender affirming, or cross-sex hormone treatment (Van de Grift et al., 2016; Hembree et al., 2017; T'Sjoen et al., 2020).

The cross-sex hormone treatment is acceptably safe over the short and medium terms regarding cardiovascular and metabolic risks (Moore et al., 2003; Gooren et al., 2008; Wierckx et al., 2012; Meriggiola and Gava, 2015) but comorbidities such as hormone dependent cancers (Gooren, 2011) and meningiomas (Bergoglio et al., 2013; Ter Wengel et al., 2016; Mancini et al., 2018) as well as increased depressive mood (Asscheman et al., 1989; Blosnich et al., 2013) and high suicide rates (Asscheman et al., 2011) were reported.

Although few previous reports have addressed the effects of the cross-sex hormone treatments in the brain of trans women, pre-post treatment structural MRI studies have shown strong effects on brain structure (Hulshoff Pol et al., 2006; Zubiaurre-Elorza et al., 2014). After four months' cross-sex hormone treatment, the authors reported decreased cerebral volumes and enlarged ventricular volumes (Hulshoff

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Pol et al., 2006). In another study, after at least six months of estrogen and antiandrogen (cyproterone acetate) treatment, trans women showed a general volumetric decrease of total gray matter, cortical thickness, thalamus and caudate as well as ventricular enlargement (Zubiaurre-Elorza et al., 2014). Ventricular volume increases were later confirmed, additionally reporting a decrease in right hippocampus volume after four months of cross-sex hormone treatment (Seiger et al., 2016). Using a cross-sectional design, after at least one year of treatment, trans women showed smaller insula volumes bilaterally than cisgender (control) men and women (Spizzirri et al., 2018). Functionally, during a mental rotation task, trans women receiving chronic hormone treatment showed a parietal hypoactivation that correlated negatively with the months of their treatment (Carrillo et al., 2010). However, despite the wealth of information accumulated on the cerebral responses to hormonal supplementation, the physiological mechanisms underlying these changes in the brain of trans women remained to be elucidated.

To address brain volume changes associated to cross-sex hormone treatment we implemented here, for the first time to our knowledge, a rat model allowing the use of invasive approaches, unavailable for ethical use in the human brain. In particular, our model allowed for a longitudinal follow up of cerebral MRI alterations, as well as brain regional sampling, providing an integrative microstructural and neurochemical characterization of the cerebral process. We hypothesized that, in the brain of the adult male rat, estradiol alone, or co-administered with an androgen receptor blocker and an antigonadotropic agent like cyproterone acetate, would reduce cortical volume. Because fractional anisotropy, a diffusion tensor imaging measure of white matter microstructure integrity and axonal coherence, increases in androgenized adult female rats (Perez-Laso et al., 2018) and trans men (Rametti et al., 2012), we predicted a decrease in male adult rats under cross-sex hormone treatment. With respect to brain metabolites, if hormone treatment affects cells water metabolism, we would expect changes in the relative concentrations of osmolyte molecules and, if the cross-sex hormone treatment induce cytotoxic effects, decreases in *N*-acetyl-aspartate (NAA) would be expected.

Briefly, our results replicated, in the rat brain, the volumetric decreases observed in trans women under cross-sex hormone treatment, maintaining or increasing fractional anisotropy by the end of the experiment and increasing the relative osmolyte concentrations in cortex and subcortical structures. Estrogens are more potent drivers of brain changes in male rats than anti-androgen treatment.

2. Materials and methods

2.1. Subjects

Thirty 60-day-old male Wistar rats (Instituto de Investigaciones Biomédicas Alberto Sols, IIB, Madrid Spain) were randomly divided into three groups: control (C; $n = 10$), estradiol (E_2; n = 10); and estradiol + cyproterone acetate (E_2 + CA; n = 10). Rats were housed three or two animals per cage, with ad libitum access to food and water in a temperature and humidity-controlled room. One control animal was excluded from the experiment and statistical analyses, since after day 15, it could not be safely fitted in the MRI probe and the focused microwave fixation system. All experimental procedures were approved by the ethical committee of IIB and performed in accordance with the EU animal welfare directive (Directive 2010/63/EU).

2.2. Experimental design

The experiment lasted 30 days (Fig. 1). Rats received daily subcutaneous injections of 0.2 mg/kg of β-estradiol 17-valerate (Sigma Aldrich, Madrid, Spain; E_2 group), 0.2 mg/kg of β Estradiol 17-valerate plus a 0.8 mg/kg dose of Cyproterone Acetate (Sigma Aldrich, Madrid, Spain; E2 + CA group) or 1.2 mg/kg propanodiol (Panreac, Barcelona, Spain; Control group). The CA dose was equivalent, and the E_2 dose a little higher than that prescribed for trans women (70 kg) under cross-sex hormone treatment (Zubiaurre-Elorza et al., 2014; Aranda et al., 2017). Cyproterone acetate is an androgen receptor blocker and an antigonadotropic agent that is administered to avoid the effects of androgens on body tissues. After vaginoplasties the administration of CA is suspended. Doses were maintained constant according to the weight of each animal. Rats were monitored three times in the course of the treatment, on days 1, 15 and 30 (D1; D15, D30).

Before MRI acquisitions, subjects were anesthetized with 2% isoflurane in 1 L of oxygen in an induction chamber, and the flow of anesthetic gas constantly regulated to maintain a breathing rate of 50 ± 20 bpm (SA Instruments, Stony Brook, NY). Body temperature was maintained at approximately at 37 °C using warm water through a heat exchanger blanket placed inside the animal platform. Immediately after anesthesia, blood samples for testosterone radioimmunoassay were taken from the tail vein before structural MRI and DTI acquisitions. On Day 30, after the experiment, animals under anesthesia, were euthanized using a system for focused microwave irradiation (Muromachi Microwave 5KW Model TMW-6402C; Toshiba, Tokyo, Japan), with a power setting of 5 kW, and an exposure time of 2.7 s. Afterward, the skull was opened, and brains dissected for ^{1}H High Resolution Magic Angle Spinning (1H HRMAS) acquisitions to investigate their neurochemical profile.

2.3. Blood sampling and hormonal essay

Plasma testosterone concentrations were measured by radioimmunoassay (RIA) using a commercial kit (DRG International Inc., Springfield, NJ07081 USA). Testosterone present in the sample, or in the calibrator, competes for a fixed number of antibody sites during the assay time with a fixed amount of ^{125}I labeled testosterone. No extraction or chromatography is required given the high specificity of the coated antibodies. After 3 hours' incubation at 37 °C, one aspiration step ends the competition reaction. The tubes are then washed with 3 ml of washing solution and aspirated again. Testosterone concentrations of the samples were determined by dose interpolation from a calibration curve containing known testosterone concentrations. The specificity of the antibody, as the percentage of cross-reaction, was estimated by comparing the concentration that produced a 50% inhibition < 0.31% for dihydrotestosterone; < 0.28% for androstenedione and < 0.004% for other compounds. The detection limit was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation, as measurements of intra- and inter-assay precision, were 4% and 5.1%, respectively.

2.4. Magnetic resonance imaging

MRI acquisitions were performed on a Bruker AVANCE III 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 in a Linux environment.

T2-weighted (T2-W) contiguous 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 μm2, slice thickness = 1.50 mm, 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 μm2 and 2 b values (100 and 1400 s/mm^2).

Finally, DTI maps of mean diffusivity (MD) and fractional

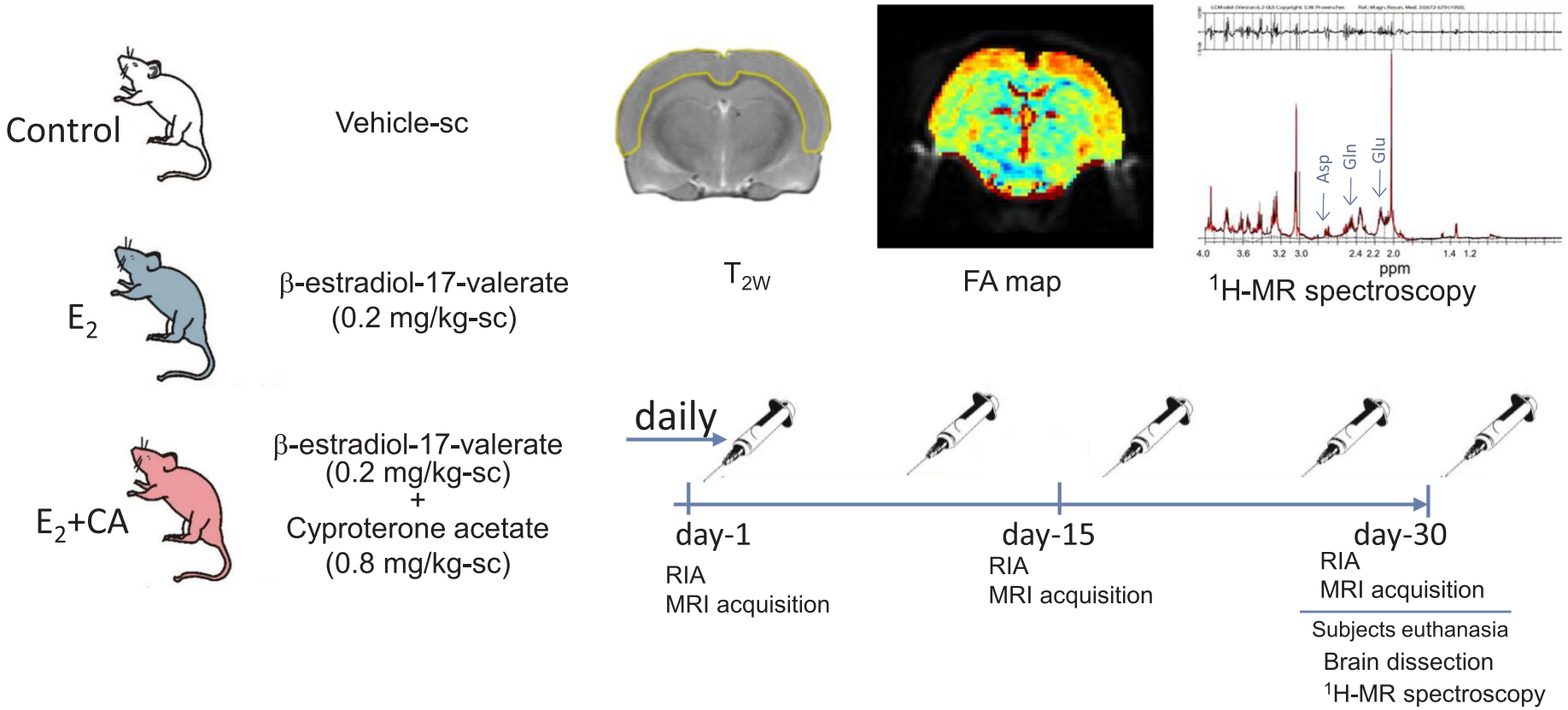

Fig. 1. Experimental design. Subjects were daily subcutaneously injected with hormone or vehicle. On days 1, 15 and 30, animals were anesthetized and blood samples for testosterone radioimmunoassay (RIA) were taken from their tails before they were placed in the scanner for brain structural MRI acquisition. Cortex is delimited by a yellow line. FA: fractional anisotropy; MRI: magnetic resonance imaging; ^{1}H MR: hydrogen (^{1}H) magnetic resonance spectroscopy; sc: subcutaneous administration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

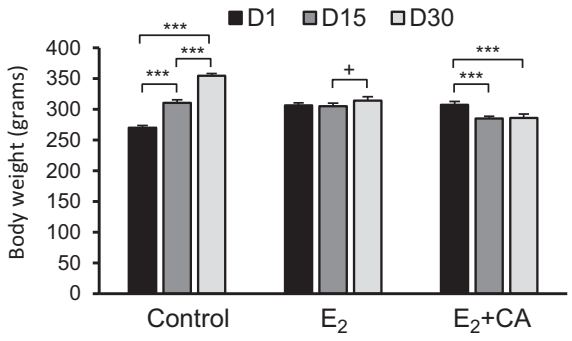

Fig. 2. Intragroup comparisons of body weight of rats on each experimental condition. Results are shown as mean ± standard error of each animal group. ***$p < 0.001$, +$p = 0.054$. E_2: estradiol group; E_2 + CA: estradiol + cyproterone acetate group. D: day of MRI acquisition.

anisotropy were obtained by a linear fitting of the logarithm of signal intensity (S) versus the b factor according to the expression: Sb = S0 exp(−ADC × b), using the homemade software MyMap Analyzer, based on MatLab v 7.0 scripts. MD and FA values were extracted from cerebral maps of the regions of interest (ROI) with Image J software (http://imagej.nih.gov/ij/; National Institute of Health).

2.5. Ex vivo ^{1}H HRMAS

The ex vivo ^{1}H MR spectra were acquired with a Bruker Advance 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. Briefly, 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 minimize 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. We collected spectra with 64 k data points, using a spectral width of 10 kHz (20 ppm), water presaturation during a 2 s relaxation delay and total acquisition time of approximately 19 min. We processed all spectra using LC Model version 6.2-OR (Stephen Provencher, Oakville, ON, Canada), a prior-knowledge based spectral fit software. We only considered metabolites fitting within a Cranmer-Rao Lower Bound limit smaller than 20%. Regions of interest (ROIS) were selected considering their estrogen receptor content (Simerly et al., 1990).

2.6. Estimation of neocortex volume

To estimate bilateral neocortex volume, we used the online ImageJ Program 1.48 for Windows (http://imagej.nih.gov/ij/; National Institute of Health). We made three series of measurements taken from the MRI slices obtained on Days 1, 15 and 30 of the experiment. We ordered contiguous MRI slices sequentially in a rostral-caudal direction, and the neocortical perimeter was outlined in every image by comparison with the corresponding plates of a brain atlas (Paxinos and Watson, 1996). Landmarks limiting the neocortical volume measurements were the rhinal fissure and the midline. The volume (mm^3) was calculated with the formula: V = Σa × h, where "a" is the measured area (mm^2) in every RM slice, and "h" the slice thickness (1.5 mm).

2.7. Statistical analyses

Statistical analyses were performed using SPSS version 24.0 (SPSS Inc., Chicago, Illinois). We conducted five sets of analyses. Repeated measures ANOVA analyses with experimental condition (control vs. E_2 vs. E_2 + CA) and day of treatment (D1 vs. D15 vs. D30) was conducted on body weights. ANOVA analyses with experimental condition (control vs. E_2 vs. E_2 + CA) was conducted on testicle weights. Repeated ANOVA tests with experimental condition and day of treatment, were conducted on the level of testosterone, total cortical volume, and left and right hemisphere cortical volume. Further repeated measures ANCOVA (controlling for total cortical volume at D1) analyses with experimental condition and day of treatment were conducted for fractional anisotropy. For metabolites, measurements from ex vivo ^{1}H HRMAS MANOVA analyses of experimental condition samples were conducted in the selected regions to control for multiple comparisons.

In those cases in which the interactions between experimental condition and day of treatment were significant, we conducted two post-hoc Bonferroni tests: 1) a first test to compare the effects of the day of treatment on each group (intragroup effect); and 2) a second test to compare each group on each day of treatment (intergroup comparison). Finally, two sets of correlational analyses were conducted. A first set of correlations between total cortical volume and metabolites, and a second test of correlations between fractional anisotropy and metabolites. Both analyses were performed separately for each of the treatments.

3. Results

3.1. Body and testicle weights

3.1.1. Body weight

The ANOVA repeated measures showed a significant condition x day of treatment interaction ($F_{(2,26)} = 198.386$, $p < 0.001$, $\eta 2 = 0.939$). Post-hoc analyses for testing intragroup effects showed three different patterns in relation to body weight gains (Fig. 2). Control rats increased their body weight significantly from D1 to D15, and from D15 to D30. No changes in weight were observed in E_2 treated subjects between D1 and D15 ($p = 0.618$) although body weight increased from D15 to D30, and there was a marginal statistical significance between D1 and D30 ($p = 0.054$). Body weight in the E_2 + CA treated group decreased significantly in the first fifteen days of the experiment ($p = 0.024$), but not from D15 to D30 ($p = 0.616$).

Inspection of the means at D1 in Fig. 2 showed no significant differences in weight between the control and the E_2 treated groups ($p = 1.000$), a marginal difference between the control group and the E_2 + CA treated group ($p = 0.057$), and no differences between the two experimental groups (E_2 and E_2 + CA groups, $p = 0.124$).

3.1.2. Testicle weights

A significant main effect for treatment day ($F_{(2,26)} = 139.639$, $p < 0.001$, $\eta 2 = 0.915$), indicated that the testicle weight was significantly higher in the control than in the E_2 ($M = 1.729$ g ± 0.043 vs. $M = 0.613$ g ± 0.082, $p < 0.001$) and the E_2 + CA ($M = 0.414$ g ± 0.040, $p < 0.001$) groups. Testicle weight in the E_2 condition was slightly higher than in the E_2 + CA condition ($p = 0.066$).

3.2. Testosterone

We tested for ANOVA invariance on D1 for the level of testosterone and found homogeneity of variance. The repeated measures ANOVA yielded a significant condition x day of treatment interaction, ($F_{(2,26)} = 12.46$, $p = 0.001$, $\eta 2 = 0.489$) (Table 1). *Post-hoc* analyses for testing intragroup effects indicated that the levels of testosterone increased significantly for the control group between D1 and D15 and decreased between D15 and D30. However, the level of testosterone remained similar between D1 and D30 ($p = 0.368$). E_2 treated rats significantly decreased their levels of testosterone from D1 to D15, but no differences were found between D15 and D30. E_2 + CA treated subjects showed decreasing levels of testosterone between D1 and D15, but no differences were found between D15 and D30.

Table 1
Testosterone levels (ng/ml) as a function of experimental condition and day of treatment.

	D1		D15		D30	
	M	SE	M	SE	M	SE
Control	1.061_b	0.270	1.901_a	0.413	1.402_b	0.463
E2	1.012_b	0.284	0.043_c	0.011	0.034_c	0.015
E2 + CA	1.215_b	0.329	0.043_c	0.023	0.007_c	0.001

D: day of collecting blood and MRI acquisition; SE: standard error; Note: means with different subscripts are different at least $p < 0.01$.

Post-hoc analyses for testing intergroup effects indicated that at D1, no significant differences were found between the three groups ($ps > 0.627$). At D15, control subjects had significantly higher levels of testosterone than the E_2 and E_2 + CA treated groups. No differences were found between the two treatment groups ($p = 0.308$). At D30, the control group had continued to have significantly higher levels of testosterone than the E_2 and E_2 + CA treated rats with no differences being found between the two treatment groups ($p = 0.345$). Testosterone was below detection limits for some animals in the two experimental groups (D15:7 E2, 7 E2 + CA; D30: 8 E2, 10 E2 + CA).

3.3. Cortical volume

3.3.1. Total cortical volume

The repeated measures analysis yielded a significant condition x day of treatment interaction ($F_{(2,26)} = 7.033$, $p = 0.004$, $\eta 2 = 0.351$), (Fig. 3A). Post-hoc analyses for intragroup effects indicated that for the control group the total cortical volume decreased significantly between D1 and D15 (Fig. 3A) but did not change between D15 and D30 ($p = 0.134$). The change between D1 and D30 was not significant ($p = 0.395$). E_2 treated rats reduced their cortical volume significantly through the experiment from D1 to D15, and from D15 to D30, while the E_2 + CA group decreased cortical volume significantly only from D1 to D 15, but not from D15 to D30, ($p = 0.710$).

Intergroup post hoc analyses indicated that at D1, E_2 animals had a higher cortical volume than control animals ($p = 0.027$) or E_2 + CA animals ($p = 0.007$). There was no significant difference between control and E_2 + CA treated subjects regarding this parameter ($p = 0.628$).

3.3.2. Right and left hemisphere volume

3.3.2.1. Right hemisphere volume. The repeated measures analysis on the right hemisphere yielded a significant condition x day of treatment interaction ($F_{(2,26)} = 5.976$, $p = 0.007$, $\eta 2 = 0.315$) (Fig. 3B). Post-hoc analyses for testing intragroup effects indicated that, in the control group, the right cortical volume did not change significantly between D1 and D15 ($p = 0.106$), or between D15 and D30 ($p = 0.180$). E_2 treated animals showed a significantly reduced right hemisphere volume between D1 and D15, continuing between D15 and D30. E_2 + CA treated animals showed a marginally decreased right hemisphere volume between D1 and D 15, but the decrease was not significant between D15 and D30 ($p = 0.257$). Nevertheless, the reduction between D1 and D30 was significant.

3.3.2.2. Left hemisphere volume. Analysis of repeated measurements for the left hemisphere also showed a significant condition x day of treatment interaction ($F_{(2,26)} = 5.696$, $p = 0.009$, $\eta 2 = 0.305$) (Fig. 3C). Post-hoc analyses of intragroup effects indicated that for control subjects the left cortical volume significantly decreased between D1 and D15 but did not change between D15 and D30 ($p = 0.155$). E_2 treated animals showed significant reductions in left hemisphere volume from D1 to D15, and from D15 to D30. E_2 + CA treated animals also showed significant decreases in left hemisphere volume between D1 and D15, ($M = 217.8$, ± 4.11 vs. $M = 208.54$, ± 4.06, $p = 0.023$), but not between D15 and D30 ($p = 0.574$).

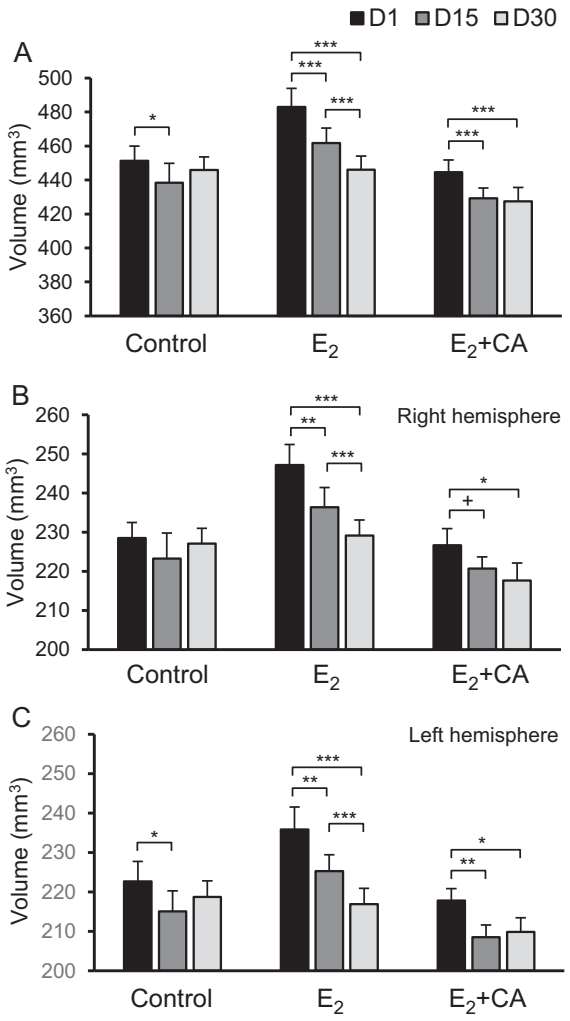

Fig. 3. Total cortical volume (A) and right (B) and left (C) cortical hemisphere volumes on each experimental condition and day of treatment. Results are shown as mean ± standard error of each animal group. +$p = 0.054$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. E2: estradiol group; E2 + CA: estradiol + cyproterone acetate group. D: day of MRI acquisition.

The fact that the significant 2-way interactions for both right and left hemispheres are similar, indicates that the same pattern of changes are produced and that this treatment does not produce a laterality effect.

3.4. Diffusion tensor imaging

Since we found intergroup differences (E_2 vs Control, and E_2 vs E_2 + CA) in total cortical volume at D1 before treatment instauration, a repeated measures ANCOVA (with total cortical volume at D1 as control variable) analyses with experimental condition and day of treatment were conducted on the total fractional anisotropy (FA), a measurement of white matter microstructure integrity and axon coherence. The analyses yielded a significant condition x day of treatment interaction ($F_{(2,25)} = 4.712$, $p = 0.018$; $\eta 2 = 0.274$) (Fig. 4). No significant variations in mean diffusivity were found.

Intragroup effects indicated that control animals showed significant increases in FA from D1 to D15 followed by significant decreases between D15 to D30. However, the difference in FA between D1 and D30 was not significant ($p = 0.358$). E_2 treated animals did not modify their

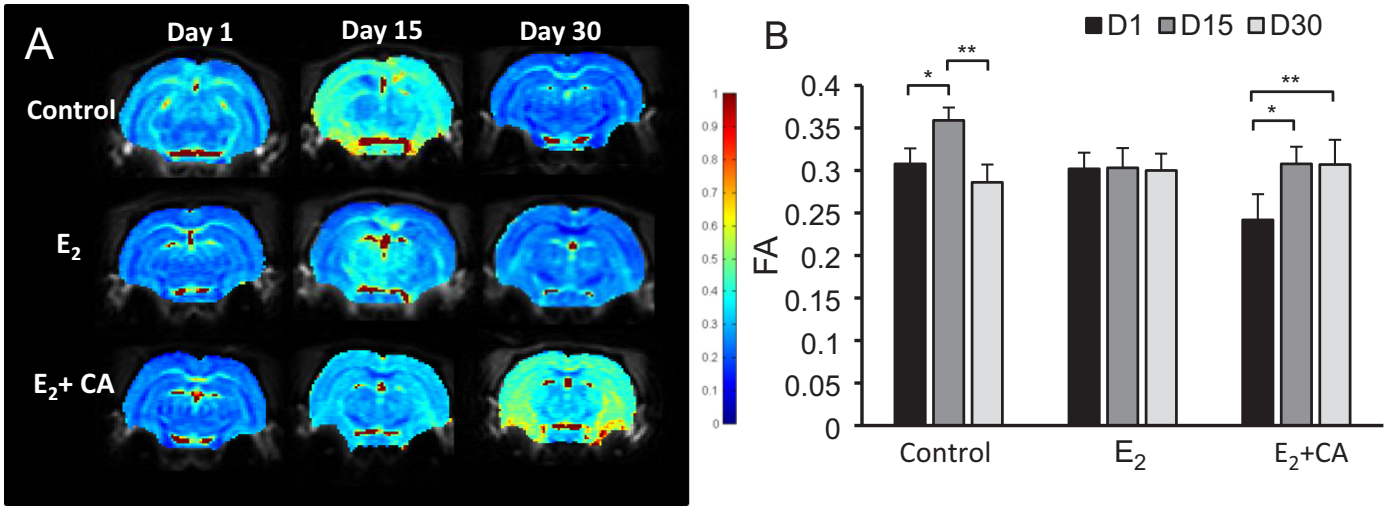

Fig. 4. Panel A shows fractional anisotropy (FA) maps of representative subjects of the three experimental groups. In panel B the intragroup analyses are plotted for total FA as a function of experimental condition and day of treatment. E_2: estradiol group; E_2 + CA: estradiol + cyproterone acetate group. D: day of MRI acquisition. $^*p < 0.05$, $^{**}p < 0.01$.

total FA either between D1 and D15 ($p = 0.515$) or between D15 and D30 ($p = 0.357$). E_2 + CA treated animals showed significantly increased total FA between D1 and D15 but not between D15 and D30, although the increase from D1 to D30 was significant.

3.5. Metabolites

We report here the results of the MANOVA analyses for Frontal Lobe, Parietal Cortex, and Hippocampus with experimental condition and post-hoc Bonferroni test (Fig. 5).

3.5.1. Frontal lobe

The significant effects found in aspartate, glutamine, and glutamate + glutamine levels indicate that GABA, glutamine and glutamate + glutamine increased their relative concentration in the E_2 condition as compared to the control condition. In contrast, aspartate decreased in E_2 as compared to the control condition. In addition, glutamine also increased in the E_2 as compared to the E_2 + CA condition.

3.5.2. Parietal cortex

The effects on GABA, NAA, choline + glycerophosphorylcholine + O-phosphocholine (Cho + GPC + PCh) were significant and were marginal for glutamine, and glutamate + glutamine, indicating that glutamine, NAA and glutamate + glutamine increased in the E_2 treatment condition, and GABA and Cho + GPC + PCh increased in the E_2 + CA treatment condition as compared to the control condition.

3.5.3. Hippocampus

Significant effects were found on glutamate and glutamate + glutamine with a marginal effect on creatine. There was a higher relative concentration of these three metabolites in E_2 rats as compared to the control condition. Glutamate was also higher in the hippocampus of E_2 + CA rats than in the control animals.

3.5.4. Hypothalamus

Significant effects were found in glutamine, glutamate, and in glutamate + glutamine. The results showed higher relative concentrations of these three metabolites in E_2 as compared to the E_2 + CA treated animals as well as a higher concentration of glutamate + glutamine than in the control condition.

3.5.5. Correlations between metabolite variations, total cortical volume and fractional anisotropy

The most significant correlations (Table 2) were from metabolites at the parietal cortex. Glutamine [r(8), −0.81, $p = 0.005$], Cho + GPC + PCh, [r(8) = −0.77, $p = 0.01$], and glutamate + glutamine, [r(8) = −0.69, $p = 0.028$] correlated negatively with the total volume of the cortex. In addition, also in parietal cortex, glutamine levels correlated negatively with fractional anisotropy values, r (8) = −0.77, $p = 0.01$. No other correlations became statistically significant.

4. Discussion

We investigate the mechanisms underlying changes in the brain of transgender women after body feminization implementing a model of pharmacologically-estrogenized adult male rats, that allows us to follow longitudinally, and in vivo, regional volume changes and white matter microstructure using MRI, as well as ex vivo metabolite profiles from different brain regions using ^{1}H HRMAS. In adult male rats, we observed: (1) E_2 and E_2 + CA administration produced a generalized decrease in the volume of the cortex that affected both hemispheres. (2) By the end of the experiment, the control group showed a tendency to decreased fractional anisotropy while the E_2 group maintained their initial values and the E_2 + CA group showed a sharp and sustained increase in fractional anisotropy; (3) E_2 treatment produced increases in the relative concentrations of NAA, GABA, glutamine, glutamate, and creatine in all the cerebral regions studied except for a decrease in aspartate that was observed in the frontal pole; there were negative correlations between the relative concentrations of glutamine in parietal cortex and total cortical volume and fractional anisotropy.

Both E_2 and E_2 + CA decreased the level of plasma testosterone and testicular weight. The reduced plasma testosterone is likely a reflection of negative feedback effects from E_2 on GnRH release that would reduce testicular weight. With respect to body weight, while the control group gained weight over the course of the experiment, E_2 subjects showed a marginal increase and the E_2 + CA group showed a significant decrease. Although we did not monitor eating, the lack of weight gain observed in the E_2 group could reflect the well-known estrogenic inhibition of eating and the loss in the E_2 + CA group could reflect an estrogenic inhibition of eating (Asarian and Geary, 2006) probably enhanced by an increase in E_2 levels resulting from the aromatization of the available testosterone. Moreover, it has been reported that CA administration increased the level of blood glucose and liver glycogen and

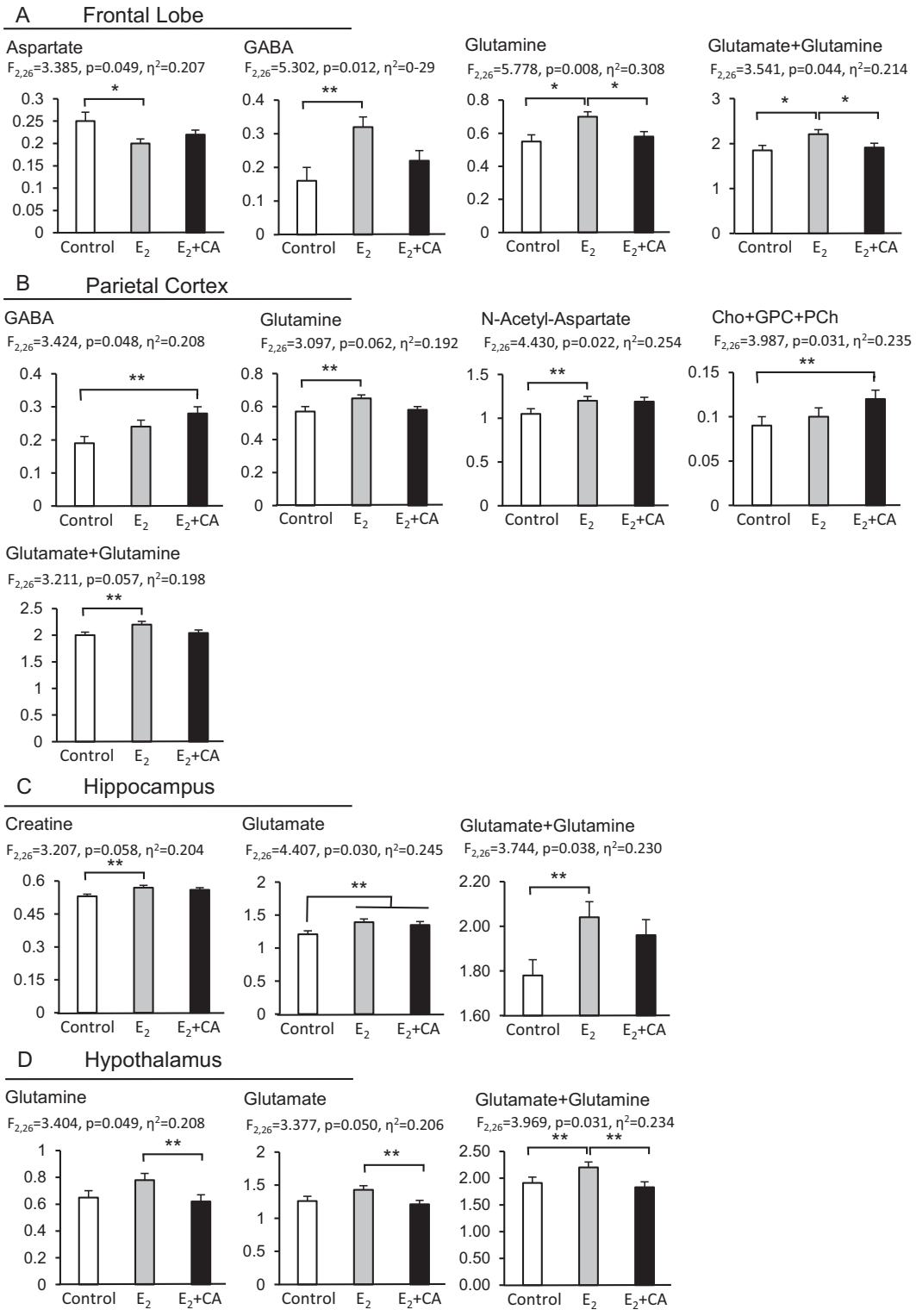

Fig. 5. Brain metabolites (relative concentrations to PCr + Cr) after pharmacological feminization treatments. PCr: Phosphocreatine; Cr: creatine; GABA: γ-aminobutyric acid; GPC: glycerophosphorylcholine; PCh: O-phosphocholine. *$p < 0.05$; **$p < 0.01$.

reduced body weight and organ weight in rats (Bhargava et al., 1981).

4.1. Cortical volume

With respect to the stage of cortical maturation, the 60-day-old subjects were adult males because rats reach the largest cortical volume at an age of two months (Mengler et al., 2014). E_2 and E_2 + CA treatments produced a decrease in total cortical volume that was equal in both hemispheres and more pronounced in the E_2 condition; this replicated in rats the same decremental effects on cortical volume observed in humans receiving cross-sex hormone treatment (Hulshoff Pol et al., 2006; Zubiaurre-Elorza et al., 2014; Seiger et al., 2016; Spizzirri et al., 2018).

We reported that, in trans women, administration of at least six

Table 2
Correlations (r) between statistically significant brain metabolites[#] and cortical volume or fractional anisotropy.

		Control		E_2		E_2 + CA	
		Total volume	FA-D30	Total volume	FA-D30	Total volume	FA-D30
Frontal lobe	Aspartate	0.28	−0.36	−0.10	0.48	−0.46	−0.08
	GABA	0.13	−0.05	−0.15	0.18	0.51	0.19
	Glutamine	0.58	0.51	−0.45	0.11	0.70*	0.28
	Glutamate + glutamine	0.44	0.46	−0.33	0.00	0.24	0.28
Parietal cortex	GABA	0.02	0.08	−0.00	−0.05	−0.70*	0.15
	Glutamine	0.35	0.10	−0.81**	−0.77**	−0.35	0.05
	N-acetil aspartate	0.42	−0.10	−0.25	−0.03	0.01	0.45
	Cho + GPC + PCh	−0.54	−0.25	−0.77**	−0.60	−0.64*	0.12
	Glutamate + glutamine	−0.19	−0.12	−0.69*	−0.35	−0.55	0.24
Hippocampus	Creatine	0.51	−0.14	0.47	0.04	0.28	−0.28
	Glutamate	0.18	−0.34	−0.40	−0.36	−0.30	0.35
	Glutamate + glutamine	0.46	−0.19	−0.50	−0.39	−0.17	0.33
Hypothalamus	Glutamine	0.51	0.11	−0.24	0.27	0.16	0.14
	Glutamate	0.25	0.35	−0.26	0.16	−0.07	0.36
	Glutamate + glutamine	0.44	0.24	−0.27	0.22	0.02	0.29

[#] Relative concentrations to PCr + Cr. PCr: phosphocreatine; Cr: creatine; GABA: γ-aminobutyric acid; GPC: glycerophosphorylcholine; PCh: O-phosphocholine; FA-D30: fractional anisotropy (mean at day 30).
* $p < 0.05$.
** $p < 0.01$.

months of estradiol valerate associated to cyproterone acetate produced a decrease in total gray matter, cortical and subcortical gray matter volumes and a global thinning of the cortex associated to enlargement of the ventricular system (Zubiaurre-Elorza et al., 2014). These findings agree with other studies on total brain volume and the volume of the hypothalamus (Hulshoff Pol et al., 2006), the hippocampus (Seiger et al., 2016) and the insula (Spizzirri et al., 2018). In women, the effects of estradiol on gray matter volume have been also observed in physiological conditions during the menstrual cycle (Protopopescu et al., 2008) and pregnancy (Oatridge et al., 2002). Moreover, women under replacement therapy receiving conjugated estrogens show decreases in cortical volume (Resnick et al., 2009; Casanova et al., 2011) like those we found in adult male rats administered estradiol valerate. Together, these evidences suggest the hormone estradiol is involved in the control of the brain volume.

It has been hypothesized that brain cortical volume decreases in trans women were due to lower testosterone levels induced by the cross-sex hormone treatment having decreased the anabolic function of testosterone (Zubiaurre-Elorza et al., 2014). E_2 and E_2 + CA rats showed a substantial decrease in the weight of the testicles and testosterone levels. Moreover, E_2 + CA animals showed a decrease in body weight contrary to the marginal weight increase observed in the E_2 group. These effects suggest a decrease of the anabolic effects in both experimental groups. The observed decreases in cortical volume could be a part of a general change in the anabolic/catabolic balance in the experimental groups.

4.2. White mater microstructure

We predicted a decrease in fractional anisotropy in hormone treated rats. We found a decrease in fractional anisotropy in the control group by the end of the experiment, suggestive of ageing (Kochunov et al., 2012). However, E_2, partially, and E_2 + CA, completely, prevented the decrease observed in the control group. Interestingly, the changes in fractional anisotropy may be closely related to those observed in cortical volume. Since a decrease in cerebral volume may impose restrictions on the average orientation in the axons, this may explain the preservation and increase in fractional anisotropy values in the hormone treated groups. Thus, increased fractional anisotropy values in experimental subjects could be derived from altered axonal rearrangements caused by the volume restrictions imposed by the treatments.

Parietal cortex fractional anisotropy negatively correlated with glutamine. This correlation should focus future attention on oligodendrocytes, since glutamine is a primarily glial metabolite. The integrity of cerebral white matter depends on axonal myelination by the oligodendrocytes. These cells contain glutamine synthetase (Fressinaud et al., 1991). The increase in the glutamine concentration with reduced fractional anisotropy might reflect a reduction of water content in these cells, and, within the axons, suggesting that white matter integrity may be affected by the cross-sex hormone treatment.

4.3. Metabolite profile

E_2 treatment resulted in important changes in the metabolite profiles of cortical and subcortical structures while E_2 + CA treatment only produced changes in cortical structures. The general increase of the relative concentrations almost of all the metabolites in E_2 treated adult male rats is consistent with the decreased volume observed in their cortex. Moreover, relative metabolite concentrations also increased in the hippocampus and the hypothalamus, suggesting that the volume of these structures also decreases under supraphysiological E_2 doses. Thus, changes in cerebral volume appear to dominate in the metabolic phenotype induced by E_2 treatment.

Since the hormonal treatments appear to affect the neurocellular water metabolism, we anticipated finding changes in cerebral osmolytes. Glutamine is a major osmolyte and an astrocyte marker (Norenberg and Martinez-Hernandez, 1979), and its relative concentration increased in cortical and subcortical structures in E_2 treated male rats. This finding confirmed that E_2 contributes considerably to the control of water and osmolyte trafficking. The change in concentration of an osmolyte like glutamine in E_2 treated male rats, and its negative correlation with total volume of the cortex supports the role of E_2 in water trafficking within brain cells. Indeed, this increased concentration of glutamine and other metabolites together with the volume reductions, suggest an active mechanism of neurocellular water extrusion induced by estradiol, explaining the volume decreases in the cortex of the adult feminized male rats.

Cerebral volume regulation is the consequence of water movements across neurocellular membranes, a process mediated by aquaporins (Nagelhus and Ottersen, 2013). It is known that Aquaporin 4 (AQP-4), the main member of the Aquaporin superfamily present in the brain, is in the astrocytic end feet, providing a crucial link between microvascular blood flow and metabolic coupling between neurons and astrocytes. Moreover, it has also been reported that E_2 modifies AQP-4

expression, supporting the relationship between E_2 and cerebral volume regulation (Rutkowsky et al., 2011). Thus, estradiol effects on metabolite concentrations mediated through AQP-4 expression are excellent candidates for future research.

However, volume changes may not be the only determinant of the alterations observed in metabolite profiles. In particular, the decrease in the relative concentration of aspartate suggests that specific metabolic regulation effects may superimpose the general volume regulation effect. Aspartate concentrations may be specifically decreased to maintain the aspartate aminotransferase equilibrium (Eq. (1)), being the most potent aminotransferase in brain, and this could balance the increases in glutamate with a decrease in aspartate (LaNoue et al., 2001).

$$K' = \frac{[\alpha - ketoglutarate][Asp]}{[Oxalacetate][Glu]}$$

Glutamine is the precursor of excitatory (glutamate, aspartate) and inhibitory (GABA) amino acids (Walls et al., 2015). Since aspartate levels decreased while those of glutamate and GABA increased, this suggests that high doses of E_2 might affect the glutamine-glutamate cycle, and the associated excitatory and inhibitory functions of theses neurotransmitters.

Finally, it has been suggested that decreases in the volume of cortical and subcortical structures might be an expression of E_2 neurotoxic effects (Zubiaurre-Elorza et al., 2014). If this were the case, we predicted a decrease in the relative concentration of *N*-acetyl-aspartate (NAA), a neuronal marker. However, NAA, like other metabolites, increased its relative concentration because of the decreased intracellular water levels. Within the temporal limits of treatments in our experimental design, we did not observe a decrease in NAA, indicating that the predicted decrease in NAA may have been counterbalanced by the increase in concentration derived from volume reduction. However, further apoptotic reductions in relative NAA concentration cannot be discounted under more prolonged hormonal treatments.

4.4. Concluding remarks

Our model mimics, in the cortex of adult male rats, the decreased volume produced by the cross-sex hormone treatment in the cortex of trans women, providing a plausible explanation of the mechanisms involved. From a closer look at our findings, an integrative hypothesis might emerge. Brain cortical volume correlated negatively with increased fractional anisotropy values and relative metabolite concentrations. These reveal a reduction in water content in brain cells, mainly in astrocytes but also in neurons and the oligodendrocytes surrounding their axons, as revealed by the increase of fractional anisotropy. Indeed, decreased cortical volume could be associated to neurocellular shrinkage, a circumstance that would disrupt the delicate metabolic equilibria among metabolite concentrations within neural cells. We report such a compensatory mechanism such for aspartate, the concentration of which is decreased to comply with the aminotransferase equilibrium.

It is normally acknowledged that, at short and medium terms, cross-sex hormone treatment of trans women is relatively safe (Gooren, 2011; Tangpricha and Den Heijer, 2017). Unfortunately, trans women's clinical follow up does not include systematic checking of brain structure and function. Several groups observed cortical and subcortical volume decreases with ventricular enlargements in trans women under cross-sex hormone treatment (Hulshoff Pol et al., 2006; Zubiaurre-Elorza et al., 2014; Seiger et al., 2016; Spizzirri et al., 2018). The findings with our male rat model suggest that cerebral volume reduction with an associated water extrusion from brain cells determines a new equilibrium in metabolite concentrations and an increase in fractional anisotropy. These circumstances merit to be kept in mind in the clinical follow-up of trans women.

Declaration of competing interest

None.

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