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**Testosterone and estradiol differentially affect cell proliferation in the subventricular zone of young adult gonadectomized male and female rats**

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Testosterone and estradiol differentially affect cell proliferation in the subventricular zone of young adult gonadectomized male and female rats

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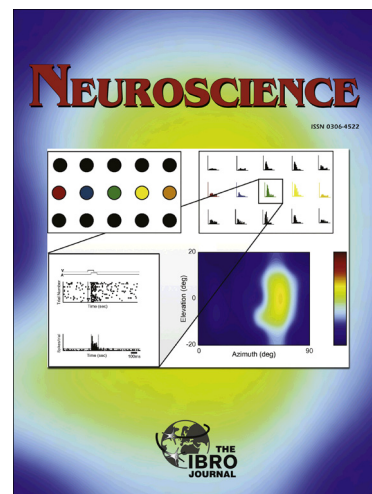
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5 **TESTOSTERONE AND ESTRADIOL DIFFERENTIALLY AFFECT CELL**  
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7 **PROLIFERATION IN THE SUBVENTRICULAR ZONE OF YOUNG ADULT**  
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9 **GONADECTOMIZED MALE AND FEMALE RATS**  
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14 **SHORT RUNNING TITLE: Sex hormones and proliferation in young adult SVZ**  
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19 **AUTHORS:**  
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**ABSTRACT**

1  
2 Steroid hormones are important players to regulate adult neurogenesis in the dentate gyrus of  
3 hippocampus, but their involvement in the regulation of the same phenomenon in the  
4 subventricular zone (SVZ) of the lateral ventricles is not completely understood. Here, in  
5 male rats, we tested the existence of activational effects of Testosterone (T) on cell  
6 proliferation in the adult SVZ. To this aim, three groups of male rats: castrated, castrated and  
7 treated with T, and controls were treated with BrdU and killed after 24 hours. The density of  
8 BrdU labeled cells was significantly lower in castrated animals in comparison to the other  
9 two groups, thus supporting a direct correlation between SVZ proliferation and levels of  
10 circulating T. To clarify whether this effect is purely androgen-dependent, or mediated by the  
11 T metabolites, estradiol (E<sub>2</sub>) and dihydrotestosterone (DHT), we evaluated SVZ proliferation  
12 in castrated males treated with E<sub>2</sub>, DHT and E<sub>2</sub>+DHT, in comparison to T- and vehicle-treated  
13 animals, and sham-operated controls. The stereological analysis demonstrated that E<sub>2</sub> and T,  
14 but not DHT, increase proliferation in the SVZ of adult male rats. Quantitative evaluation of  
15 cells expressing the endogenous marker of cell proliferation PHH3, or the marker of highly  
16 dividing SVZ progenitors Mash1, indicated the effect of T/E<sub>2</sub> is mostly restricted to SVZ  
17 proliferating progenitors. The same experimental protocol was repeated on ovariectomized  
18 female rats treated with E<sub>2</sub> or T. In this case, no statistically significant difference was found  
19 among groups. Overall, our results clearly show that the gonadal hormones T and E<sub>2</sub>  
20 represent important mediators of cell proliferation in the adult SVZ. Moreover, we show that  
21 such effect is restricted to males, supporting adult neurogenesis in rats is a process  
22 differentially modulated in the two sexes.

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**Key Words:** adult neurogenesis, gonadal hormones, testosterone, estradiol, SVZ proliferation, sex dimorphism.

## 1. INTRODUCTION

1  
2 Neurogenesis in adult mammals is mostly restricted to the so-called “adult germinative  
3 regions”: the dentate gyrus (DG) of hippocampus and the subventricular zone (SVZ)-  
4 olfactory bulb (OB) system (Bonfanti and Peretto, 2011, Tong and Alvarez-Buylla, 2014,  
5 Vadodaria and Gage, 2014). Studies performed over the last decade have shown that  
6 integration of new neurons into the DG and OB circuits optimizes key brain functions (Sahay  
7 et al., 2011), including the analysis of social stimuli underlying the reproductive behavior  
8 (Feierstein, 2012, Larsen and Grattan, 2012). Accordingly, adult neurogenesis is finely tuned  
9 by a complex interplay between extrinsic and intrinsic factors, which regulate the  
10 proliferation of neuronal progenitors in the germinative niches, as well as the migration and  
11 survival/integration of newborn neurons into the mature circuits (Lenington et al., 2003,  
12 Abrous et al., 2005, Zhao et al., 2008). Gonadal hormones have been indicated as important  
13 intrinsic regulative factors of adult neurogenesis (Galea, 2008, Galea et al., 2013). The vast  
14 majority of the studies which have investigated the role of these hormones on adult  
15 neurogenesis has been performed in the DG of hippocampus (Galea, 2008, Galea et al.,  
16 2013), whereas only a few of them have addressed this issue in the SVZ neurogenic niche  
17 (Smith et al., 2001, Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker, 2011, Tatar et  
18 al., 2013).

19 In the rat DG, gonadal hormones modulate adult neurogenesis in a rather complex way by  
20 differentially affecting cell proliferation and survival in males and females (Barker and Galea,  
21 2008). For example, in female rats each phase of the estrous cycle shows a different level of  
22 cell proliferation, peaking during the proestrus (when estrogens' levels are highest) and  
23 decreasing in estrus or in diestrus (Tanapat et al., 1999). Ovariectomy induces a decrease of  
24 cell proliferation, whereas acute treatment with estradiol (E<sub>2</sub>) to ovariectomized animals  
25 increases the number of newborn cells (Tanapat et al., 2005). By contrast, in male rats,  
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1 androgens [testosterone (T) and dihydrotestosterone (DHT)], but not E<sub>2</sub>, enhance cell survival  
2 in the DG (Spritzer and Galea, 2007).  
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4 In the SVZ neurogenic niche such studies have been mostly performed in females by  
5 specifically focusing on the effect of E<sub>2</sub> in both naïve and neurodegenerative models (Saravia  
6 et al., 2004, Hoyk et al., 2006, Suzuki et al., 2007, Brock et al., 2010, Veyrac and Bakker,  
7 2011). Only indirect evidences indicate that other gonadal hormones, such as progesterone  
8 and T can regulate neurogenesis in the adult SVZ niche (Peretto et al., 2001, Giachino et al.,  
9 2004, Zhang et al., 2010, Tatar et al., 2013). Similarly to the DG, the modulatory effect of  
10 gonadal hormones, particularly of E<sub>2</sub>, on SVZ neurogenesis appears highly variable  
11 depending on type of treatment/dose (chronic vs. acute), sex, species, and (in mouse) strain  
12 considered (Smith et al., 2001, Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker,  
13 2011, Tatar et al., 2013). In addition, such modulation differentially impacts the neurogenic  
14 process influencing progenitor cells proliferation and/or integration of newborn neurons in  
15 both the main and the accessory OB (Hoyk et al., 2006, Brock et al., 2010, Veyrac and  
16 Bakker, 2011).  
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18 Here, for the first time we specifically address the possible impact of T in the regulation of  
19 SVZ neurogenesis in the adult rat, focusing on the issue of cell proliferation. In addition, by  
20 considering that T in the brain is metabolized into E<sub>2</sub> and DHT (Celotti et al., 1991), we  
21 compared the effects exerted by exogenous treatment of acute doses of T and its metabolites  
22 in multiple groups of gonadectomized and control animals on both sexes. Our results show  
23 that the levels of circulating T and its metabolite E<sub>2</sub>, but not DHT, differentially influence cell  
24 proliferation in the SVZ of male and female rats. These data further confirm the role of  
25 gonadal hormones as important regulative factors of adult SVZ neurogenesis, and definitively  
26 demonstrate the involvement of T in such process.  
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## 2. MATERIALS AND METHODS

### 2.1. Animals

Young male (N=57) and female (N=28) Wistar rats (21 days old) were purchased from Harlan Italy (Udine, Italy) and held at the animal facility of the Department of Neuroscience, University of Turin. Animals of the same sex were maintained five per cage with a 12:12 light/dark cycle. Food and water were provided ad libitum (standard chow 4RF25-GLP with certificated non-detectable estrogenic activity, i.e. lower than 20 $\mu$ g/kg of DES equivalent, Mucedola srl, Settimo Milanese, Italy). Animal care and handling were performed according to the European Community Council Directive (86 / 609 / EEC), the Italian government institutional guidelines on animal welfare (DDL 116/92). The experimental protocol was approved by the Bioethics and Animal Welfare Committee of the University of Torino and the Italian Ministry of Health.

### 2.2. Surgery

One week after the housing of animals in the animal facility, at the age of 28 days, we performed surgical procedures by using aseptic technique. At this age, rats are in the prepuberal period and the level of testosterone (in males) is extremely low, while the level of estradiol (in females) is not increased as it will do in the following weeks (Vetter-O'Hagen and Spear, 2012). Gonadectomy after puberty may determine a sudden decrease from high to low estradiol or testosterone levels, whereas, at the prepuberty time the impact on the endocrine balance of individuals is less drastic. Male and female rats were deeply anaesthetized with 3% isoflurane vaporized in O<sub>2</sub> / N<sub>2</sub>O (30:70). Animals were randomly assigned to either bilaterally gonadectomized or sham operated groups. In males assigned to the castrated group both testes were removed by a small incision in the scrotum, followed by the blockage of the spermatic cord with a silk suture to prevent hemorrhage during dissection

1 of testis. In males assigned to the sham operated group a small incision was performed in the  
2 scrotum and then sutured without removing the testes.  
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4 In females assigned to the ovariectomized group both ovaries were removed by two small  
5 incisions on the back: the uterus was clamped near the ovary with a silk suture to prevent  
6 hemorrhage and each ovary was removed with a single cut of the oviductum near the ovary.  
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8 In the females of sham-operated group, animals received two small incisions on the back, as  
9 in the previous group, but without removing ovaries. Rats (randomly assigned 5 per cage)  
10 were then allowed to recovery for two weeks before further treatments.  
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## 22 **2.3. Procedure**

### 23 **2.3.1. Hormonal treatments**

24 *2.3.1.1. Experiment 1: Testosterone effect in male rats.* Three groups of 42 day-old animals  
25 were used: Castrated (CX, N = 5), Castrated + Testosterone (CX+T, N = 5) and control  
26 animals (SHAM, N = 5). The doses of steroid hormones for this and the following  
27 experiments were chosen according to (Spritzer and Galea, 2007). Rats belonging to the  
28 CX+T group received one intraperitoneal (i.p.) injection of T (1.00 mg/0,1 mL, Sigma-  
29 Aldrich, Milan, Italy) dissolved in sesame oil (Sigma-Aldrich, Milan, Italy), while one i.p.  
30 injection of Sesame Oil (0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy) was performed on  
31 the CX and SHAM groups.  
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50 *2.3.1.2. Experiment 2: Testosterone metabolites effect in male rat.*

51 Six groups of 42 day-old male rats were used: 1- Castrated + T (CX+T, N = 7); 2- castrated +  
52 E<sub>2</sub> (CX+E<sub>2</sub>, N = 8); 3- castrated + DHT (CX+DHT, N = 8); 4- castrated + E<sub>2</sub> + DHT (CX+  
53 E<sub>2</sub>+DHT, N = 8); 5-castrated + sesame oil (CX+OIL, N = 7); 6- sham operated animals +  
54 sesame oil (SHAM, N = 7). All animals received one i.p. injection of hormones or vehicle  
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(sesame oil) at the following concentrations: T (1.00 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy); E<sub>2</sub> ( $\beta$ -Estradiol-3-benzoate, 0.020 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy); DHT (Androstanolone, 0.5 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy) and vehicle (0.1 mL of sesame Oil; Sigma-Aldrich, Milan, Italy).

### 2.3.1.3. Experiment 3: Testosterone and estradiol effect in female rat.

Four groups of 42 day-old female rats were used: 1- ovariectomized + vehicle (OVX+OIL, N = 6) (0.1 mL of Sesame Oil; Sigma-Aldrich, Milan, Italy); 2- ovariectomized + E<sub>2</sub> (OVX+E<sub>2</sub>, N = 5) ( $\beta$ -Estradiol-3-benzoate , 0.020 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy); 3- ovariectomized +T (OVX+T, N = 5) (1.00 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy); and finally, 4- sham operated + sesame oil (SHAM, N = 4).

### 2.3.2. BrdU injections

12 hours after hormones' or vehicle's administration, all animals were i.p. injected with 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, Milan, Italy) in 0.1M Tris (pH 7.4) twice (delay 6 hours, 50 mg/kg body weight, delay 6 hours, 50 mg/kg body weight, to maximize the labeling of proliferating cells, Ponti et al., 2013). The female rats of the sham-operated group were injected all in the estrus phase of the estrous cycle.

### 2.4. Tissue preparation and immunocytochemistry

24 hours after the last BrdU administration, animals were deeply anaesthetized with an i.p. injection of a ketamine (100 mg/Kg of body weight, Ketavet, Gellini, Italy) and xylazine (10 mg/Kg of body weight, Rompun, Bayer, Germany) solution and intracardially perfused with physiological solution (NaCl, 0.9%), followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were post-fixed for 24 hours in the same fixative,

1 cryoprotected in increasing sucrose solutions and frozen in isopentane pre-cooled in dry ice at  
2 -30°C / -40°C. Brains were stored at -80°C up to the day before cryosectioning when they  
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4 were moved to a -20°C freezer. Serial free-floating 40 µm-thick coronal sections were  
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6 collected in multiwell dishes.  
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9 For BrdU immunostaining sections were treated with 2 M HCl for 1 h at 37°C, neutralized  
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11 with borate buffer pH 8.5, and subsequently incubated overnight with the primary rat anti-  
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13 BrdU antibody (AbD Serotec, Nottingham, UK, cat. #: OBT0030CX, (Schutte et al., 1987)  
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15 diluted 1:10,000, with PBS-T (Triton-X100, 0.2% in 0.1 M phosphate buffer saline, PBS)  
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17 followed by a biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-  
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19 MC15), diluted 1:250 with PBS-T. Sections were then processed for 1 hour in Avidin-Biotin  
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21 Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase activity was  
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23 visualized with a solution containing 0.400 mg/ml of 3,3'-diamino-benzidine (DAB, Sigma,  
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25 Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6.  
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31 For the detection of the phosphorylated form of Histone H3 (PHH3) and Mash1, sections  
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33 were incubated for 48 h at 4°C with anti-PHH3, 1:4000 (Millipore, Massachusetts, USA, cat.  
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35 #: 06-570 (Fukushima et al., 2009) and anti-Mash1, 1:2000 (BD Bioscience, New Jersey,  
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37 USA, cat. #: 556604 (Doetsch et al., 1997), in a solution of PBS, pH 7.4, containing 2%  
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39 Triton X-100 (Merck, Darmstadt, Germany), 0,5 % Normal Goat Serum (Vector  
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41 Laboratories, California, USA), and 2% BSA (Sigma-Aldrich, Milan, Italy). Sections were  
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43 washed and incubated, respectively, with solutions of secondary anti-rabbit 488, 1:400  
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45 (Jackson ImmunoResearch Laboratories, Pennsylvania, USA, cat. #: 11-545-144) and with  
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47 anti-Mouse 647, 1:400 (Jackson ImmunoResearch Laboratories, Pennsylvania, USA, cat. #:  
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49 115-605-146). Sections were then cover slipped with antifade mounting medium Mowiol  
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51 (Sigma-Aldrich, Milan, Italy) and analyzed with a laser scanning Leica TCS SP5 (Leica  
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53 Microsystems) confocal microscope. Images were processed using Image J  
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(<http://rsb.info.nih.gov/ij/>) and Adobe Photoshop CS3 (Adobe Systems). Only general adjustments to color, contrast, and brightness were made.

Methodological controls included omission and/or replacement of the primary antibody with an equivalent concentration of normal serum (negative controls), and omission of the secondary antibody.

## 2.5. Quantitative analyses

The number of BrdU+ cells was stereologically evaluated in six serial sections for each animal, localized at an intermediate rostro-caudal level of SVZ (about from 1.60 mm to 0.70 mm anterior to Bregma; (Paxinos and Watson, 1998). In the first experiment, the number of BrdU+ cells was evaluated in three distinct SVZ regions: the dorsal area and the medial and lateral wall of the lateral ventricle that were manually traced as indicated in Fig. 1A. In the second and the third experiment, the quantitative analysis was performed only in the lateral wall region. Sections were visualized using a Nikon Eclipse E600 microscope equipped with a motorized stage controller. Stereological estimation of the number of BrdU+ cells was performed according to the optical fractionator method (Gundersen et al., 1999) by using the Stereoinvestigator software package (MicroBrightField, Williston, VT). We traced the outline of the area of interest on all the analyzed sections and the software calculated directly the volume using the Cavalieri's methods (Gundersen et al., 1988).

For each group we calculated the density of BrdU+ cells as a ratio among the number of BrdU+ cells counted within the considered volume ( $\mu\text{m}^3$ ), the values are expressed as mean

□ Standard Error for  $10^4 \mu\text{m}^3$ .

The number of PHH3+ and Mash1+ cells was evaluated in the same way within the SVZ lateral wall. The density values of PHH3+ and of Mash1+ cells are expressed as mean,  $\pm$  Standard Error multiplied respectively, for  $10^8 \mu\text{m}^3$  (PHH3) and for  $10^5 \mu\text{m}^3$  (Mash1).

## 2.6. Statistical Analysis

The density values were analyzed via one-way ANOVA, after verifying the normality of the data, with the experimental treatment as independent variable and the density of BrdU+, PHH3+, or Mash1+ cells as dependent variable, followed, if significant, by a post-hoc analysis with the Tukey post-hoc test. The SPSS 22.0 program was used for calculating probability value.

## 3. RESULTS

### 3.1. Testosterone influences proliferation in the SVZ of prepuberal castrated male rat

In order to evaluate whether T influences proliferation in the SVZ of male rats we compared the density of BrdU+ cells in castrated (CX, N=5), castrated and treated with T (CX+T, N=5) and control (SHAM, N=5) males, 24 hours after the last BrdU injection. Cell quantification (N=6 sections/animal) was performed in three different regions of the SVZ, defined as dorsal area, medial and lateral wall (see methods and Fig.1A for details).

In the lateral wall, the density of BrdU+ cells was lower in CX animals ( $2.193 \pm 0.366$ , N=5) in comparison to both control ( $4.202 \pm 0.612$ , N=5) and CX+T ( $4.128 \pm 0.402$ , N=5) males. The one-way ANOVA showed a significant effect of treatment ( $F_{(2,12)} = 5.819$ ,  $p < 0.05$ ), and the post-hoc comparisons (Tukey test) demonstrated a significant difference among control and CX ( $p < 0.05$ ), and CX+T and CX ( $p < 0.05$ ) groups (Fig.1B). No differences were found between the CX+T animals and controls. In the dorsal area and in the medial wall of SVZ, the one-way ANOVA has not shown any significant effect of the treatment (Dorsal area:  $F_{(2,12)} = 2.542$ ,  $p > 0.05$ ; Medial wall:  $F_{(2,12)} = 0.909$ ,  $p > 0.05$ )(Fig.1C-D).

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These data indicate that castration during prepuberal period induces a significant decrease of cell proliferation in a specific region of SVZ, the lateral wall of lateral ventricle of male rats, and this decrease is restored by a treatment with T.

### 3.2. T and E<sub>2</sub>, but not DHT enhance cell proliferation in the SVZ of prepuberal castrated male

In order to investigate whether this region-specific T-dependent proliferation in the SVZ of male rat is mediated by either one or both T metabolites, E<sub>2</sub> and DHT, we treated CX animals with acute doses of T, E<sub>2</sub>, DHT, E<sub>2</sub>+DHT or vehicle (OIL). Then, we evaluated the density of proliferating cells in the SVZ following the same protocol used in the first experiment, focusing on the lateral wall of the lateral ventricle. The one-way ANOVA revealed a strongly significant effect of treatment ( $F_{(5,39)}=15.977$ ;  $p<0.001$ ) and the post-hoc comparisons demonstrated that the density of BrdU+ cells in SHAM operated males ( $3.753\pm 0.620$ , N=7) was not significantly different ( $p>0.05$ ) from CX+T ( $6.290\pm 0.752$ , N=7), CX+E<sub>2</sub> ( $5.054\pm 0.693$ , N=8), and CX+DHT+E<sub>2</sub> ( $4.814\pm 0.405$ , N=8). By contrast, this number was significantly lower ( $p<0.05$ ) in CX+OIL ( $1.400\pm 0.054$ , N=7) and CX+DHT ( $1.232\pm 0.108$ , N=8) animals in comparison to SHAM group (Fig. 2-3). Overall these data indicate that the decrease in the cell proliferation observed in animals castrated during prepuberal period is due to the lack of E<sub>2</sub>, and not to the lack of a pure androgen as DHT.

To investigate the nature of SVZ cell types involved in such T/E<sub>2</sub>-dependent response, we quantified in the SVZ lateral wall the density of cells expressing PHH3, which allows the identification of proliferating cells (Fukushima et al., 2009) and the expression of Mash1, which identifies the highly proliferating transit amplifying Type C cells (Doetsch et al., 1997). The one way ANOVA for PHH3+ cell density reported a significant effect of treatments ( $F_{(3,16)}= 6.506$ ,  $p<0.05$ ). The post-hoc analysis demonstrated that the density of

1 positive cells in CX+OIL group ( $1.805 \pm 0.538$ , N=5) was significantly lower ( $p < 0.05$ ) than  
2 the other three groups (CX+T=  $7.889 \pm 0.625$ , N=6, CX+E2=  $5.552 \pm 0.251$ , N=4, SHAM=  
3  $7.700 \pm 1.033$ , N=5)(Fig. 4A).

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6 Similarly, the one-way ANOVA for Mash1+ cell density revealed a significant effect of  
7 treatment ( $F_{(3,8)}=7.023$ ;  $p < 0.05$ ). The post-hoc analysis showed that the density of positive  
8 cells in CX+OIL group ( $3.134 \pm 0.395$ , N=3) was significantly lower ( $p < 0.05$ ) than the other  
9 three groups (CX+T=  $7.071 \pm 0.902$ , N=3, CX+E2=  $6.871 \pm 0.578$ , N=3, SHAM=  $6.761 \pm$   
10  $0.854$ , N=3)(Fig. 4B). These data indicate that T/E<sub>2</sub> control the proliferation rate of SVZ  
11 progenitors.  
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### 24 **3.3. Gonadal hormones T and E<sub>2</sub> do not regulate the SVZ proliferation in prepuberal** 25 **ovariectomized female rat**

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27 In the third experiment we investigated whether, similarly to males, T and/or E<sub>2</sub> may  
28 influence the rate of SVZ proliferation also in ovariectomized female rats. Therefore, by  
29 using the above reported methodological approach, we performed an additional experiment in  
30 females by examining cell proliferation within the SVZ lateral wall in the following groups:  
31 ovariectomized (OVX) animals, sham controls (SHAM), OVX females treated with acute  
32 doses of T (OVX+T) or with E<sub>2</sub> (OVX+E<sub>2</sub>). The density of BrdU+ cells measured through the  
33 stereological analysis was not strongly different among the experimental groups (OVX =  
34  $3.072 \pm 0.425$ , N=6, OVX+T=  $4.101 \pm 1.116$ , N=5, OVX+E<sub>2</sub>=  $3.805 \pm 1.097$ , N=5,  
35 SHAM=  $2.479 \pm 0.841$ , N=4). The one-way ANOVA ( $F_{(3,16)}= 0.618$ ,  $p > 0.05$ ) confirmed the  
36 lack of significant effect of treatments (Fig. 5). Overall, these results indicate that in contrast  
37 to males, the acute treatment with T or E<sub>2</sub> does not influence the proliferation in the SVZ of  
38 females ovariectomized in prepuberal period and show the occurrence of a novel sex  
39 dimorphism related to the regulation of cell proliferation in the SVZ region of rats.  
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#### 4. Discussion

Here we report a direct proof that the level of circulating T may influence cell proliferation in a subregion of the SVZ of male rats. In addition, we show that this activity is likely to be mediated by the aromatic metabolite E<sub>2</sub> and not by the androgenic metabolite DHT. By contrast, we demonstrate that, in the same experimental conditions, T or E<sub>2</sub> does not affect SVZ proliferation in females indicating a sex-specific regulation of neurogenesis by these hormones in the SVZ of adult rats. In addition, we demonstrate that this modulatory effect mostly involves SVZ proliferating progenitor cells, since T and E<sub>2</sub> treatment in prepuberal castrated animals increases both the PHH3 proliferating cells and the Mash1 positive Type C cells.

Interestingly, the action of T/E<sub>2</sub> is site specific and restricted to the lateral wall of the ventricle. This result is intriguing since recent data indicate that SVZ progenitor cells are organized in multiple microdomains that correlate with the expression domain of different transcription factors, and in turn give rise to different types of OB interneurons (Merkle et al., 2007, Merkle et al., 2014). Therefore, we can speculate that T/E<sub>2</sub> may influence the genesis of specific OB interneurons.

The large majority of the studies investigating the effects of steroids on adult neurogenesis has been focused on the DG of hippocampus. In this region, adrenal and gonadal steroids act as potent regulators of adult neurogenesis affecting proliferation of neural precursor cells and/or survival of newborn neurons (Galea et al., 2013). Most studies have dealt with the modulatory role of steroids (in particular estrogens) in females, whereas only a few studies have addressed the effect of androgens in males (Spritzer and Galea, 2007, Spritzer et al., 2011). Here we focused on the SVZ neurogenic niche, which generate newborn neurons fated to the OB region (Tong and Alvarez-Buylla, 2014, Hamson et al., 2013). Surprisingly, only a

1 few studies are at the moment available concerning the impact of gonadal hormones on OB  
2 neurogenesis (Smith et al., 2001, Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker,  
3 2011), despite their contribution to olfactory reproductive behavior (Keller et al., 2009), and  
4 recent data supporting a link between OB neurogenesis and reproduction (reviewed in Peretto  
5 and Paredes, 2014).  
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11 We found that two weeks after gonadectomy, a condition that virtually implies absence of  
12 significant levels of circulating sex hormones (Kashiwagi et al., 2005), the amount of cell  
13 proliferation in the SVZ, evaluated 24 hours after the BrdU treatment, was significantly  
14 reduced in comparison to intact or sham-operated animals. Moreover, we found that a single  
15 injection of T restores in short time (24 hours) the level of cell proliferation. To date, this is  
16 the first direct evidence demonstrating that in male rats the lack of T affects adult  
17 neurogenesis in the SVZ, by reducing the rate of cell proliferation.  
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29 It has been reported that gonadectomy affects adult neurogenesis also in mice, but with an  
30 opposite effect, that is, increasing cell proliferation in SVZ, however these effects are strain-  
31 and sex-dependent (Tatar et al., 2013). Finally, in the Sprague Dawley rat, androgens enhance  
32 adult neurogenesis in DG, promoting cell survival rather than proliferation (Spritzer and  
33 Galea, 2007, Hamson et al., 2013). The results of the first experiment prompted us to  
34 investigate whether T-dependent proliferation in male rat SVZ was mediated by either or both  
35 T metabolites: E<sub>2</sub> and DHT. Indeed, in the brain both metabolites are present due to the action  
36 of the enzymes aromatase (converting T into E<sub>2</sub>) and 5 $\alpha$ -reductase (converting T into DHT)  
37 (Celotti et al., 1991).  
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51 We found that acute administration of E<sub>2</sub> but not DHT was effective in restoring SVZ  
52 proliferation in castrated males. This result indicates that the proliferative activity elicited by  
53 T in the SVZ is mediated by its conversion in E<sub>2</sub>. In contrast, in the rat DG, chronic  
54 administration of T or DHT, but not of E<sub>2</sub>, was found to modulate hippocampal neurogenesis  
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1 (Spritzer and Galea, 2007). However, such effect was due to enhanced cell survival rather  
2 than proliferation. Thus, since our study was restricted to only short-survival times after  
3 BrdU administration (i.e., proliferation), further analyses are necessary to establish whether  
4 diverse gonadal hormones (e.g., DHT) differentially affect the two niches, and/or specific  
5 steps of the SVZ neurogenic process (i.e., migration and differentiation).  
6

7 Estrogens may exert protective effects under pathological conditions in several brain regions  
8 (Garcia-Segura et al., 2001, Behl, 2002) including adult neurogenic regions. An estrogen-  
9 dependent modulation of DG and SVZ neurogenesis in males was found in a mice model of  
10 induced diabetes. In these animals, E<sub>2</sub> restores the rate of SVZ proliferation in streptozotocin-  
11 diabetic male mice, contrasting the reduction of cell proliferation observed in non-E<sub>2</sub> treated  
12 males (Saravia et al., 2004). However, E<sub>2</sub> is not inducing any increase in control animals, thus  
13 supporting the hypothesis that, in male mice, E<sub>2</sub> can stimulate SVZ proliferation only in  
14 pathological conditions.  
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16 Overall, these results underline the importance of carefully consider the species, the region  
17 and type/nature of progenitors and the experimental condition, including the age at the time  
18 of gonadectomy as well as that at the time of hormonal treatments, when studying the effect  
19 of such hormones in the regulation of the two adult neurogenic niches (Galea et al., 2013).  
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21 Interestingly, in our study, the SVZ population shows a sexually dimorphic sensitivity to  
22 gonadal hormones. In fact, we found that both ovariectomy and acute administration of T or  
23 E<sub>2</sub> in OVX females did not modify the number of cycling cells in the SVZ neurogenic niche.  
24 This sex difference is not limited to SVZ: in the DG, a sex-dependent regulation of adult  
25 neurogenesis has been already described in different rodent species (for a recent review see  
26 (Galea et al., 2013).  
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28 The way by which E<sub>2</sub> interacts with SVZ in female is dependent on the species, dose- and  
29 time of administration. For example, in the prairie vole, estrous induction, which in this  
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1 species is driven by exposure to unfamiliar male chemosensory cues (Richmond and  
2 Conaway, 1969), is associated to an estrogen-dependent increase of SVZ cell proliferation  
3  
4 (Smith et al., 2001). By contrast, in C57Bl/6j/sv129 female mice chronically exposed to E<sub>2</sub>,  
5 newborn cell survival in the OB decreases and SVZ cell proliferation is not modified (Veyrac  
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7 and Bakker, 2011). On the contrary, a short-term exposure to E<sub>2</sub> negatively affects cell  
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9 proliferation in C57Bl6/J female mice SVZ (Brock et al., 2010). Interestingly, in OVX female  
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11 mice of the same strain, a chronic treatment with low doses of E<sub>2</sub> stimulates cell proliferation  
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13 in the SVZ of post-stroke animals, although such increase was not present in control animals  
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15 (Suzuki et al., 2007). In rats, a chronic treatment with E<sub>2</sub> on OVX females results in a minor  
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17 number of newborn neurons integrating in the accessory OB (Hoyk et al., 2006), however, in  
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19 this study neurogenesis was evaluated only at long survival time after BrdU injection, thus it  
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21 is not clear if this is an effect on cell proliferation or on cell survival. Our data indicate that  
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23 prepuberal ovariectomy, as well as short-term exposure to E<sub>2</sub>, does not affect the SVZ  
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25 proliferation in female rat.  
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29 However, estrogen receptors are expressed in vivo and in vitro in cells originated, or located  
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31 in the rat SVZ lining the lateral ventricles (Brannvall et al., 2005, Isgor and Watson, 2005),  
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33 thus long term effects of E<sub>2</sub> in females (e.g., increasing the responsiveness of newborn  
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35 neurons during their survival/differentiation in the OB) cannot be ruled out.  
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39 In conclusion, we think that our results, although not definitive, add a small but important tile  
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41 in the complex emerging picture describing the impact of gonadal hormones on adult  
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43 neurogenesis. Future studies, besides filling the still existing gaps of knowledge related to  
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45 this specific issue (e.g., expression analysis of gonadal hormone receptors in different SVZ  
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47 neurogenic niche cell types in males and females; role of T and or E<sub>2</sub> in mediating  
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49 integration/survival of newborn neurons in both sexes) should be directed to unravel whether  
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51 a reciprocal interplay exists between gonadal hormones, adult neurogenesis, and other  
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1 hypothalamic neuroendocrine signals/nuclei involved in the control the gonadal hormones  
2 secretion.  
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7 **5. Acknowledgments.** This work was supported by the Fondazione Cavalieri Ottolenghi and  
8  
9 by grants of the University of Torino.  
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ACCEPTED MANUSCRIPT

**Legend to the figures**

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2  
3 **Fig.1** - Variations in the density of BrdU+ cells in the SVZ of castrated males treated with  
4 testosterone. **A.** Representative section of the SVZ from a sham-operated male. The  
5 rectangles show the 3 regions that were measured in Experiment 1. LV=lateral ventricle,  
6 DA=dorsal area, MW=medial wall, LW=lateral wall, scale bar=300  $\mu\text{m}$ . **B-C.** Histograms  
7 illustrating the variations of the total number of BrdU labeled cells (Mean $\pm$ SE) in the lateral  
8 wall (B), in the medial wall (C) and in the dorsal area (D). The significant differences  
9 (ANOVA followed by Tukey test at a level of  $P < 0.05$ ) are denoted by a or b.  
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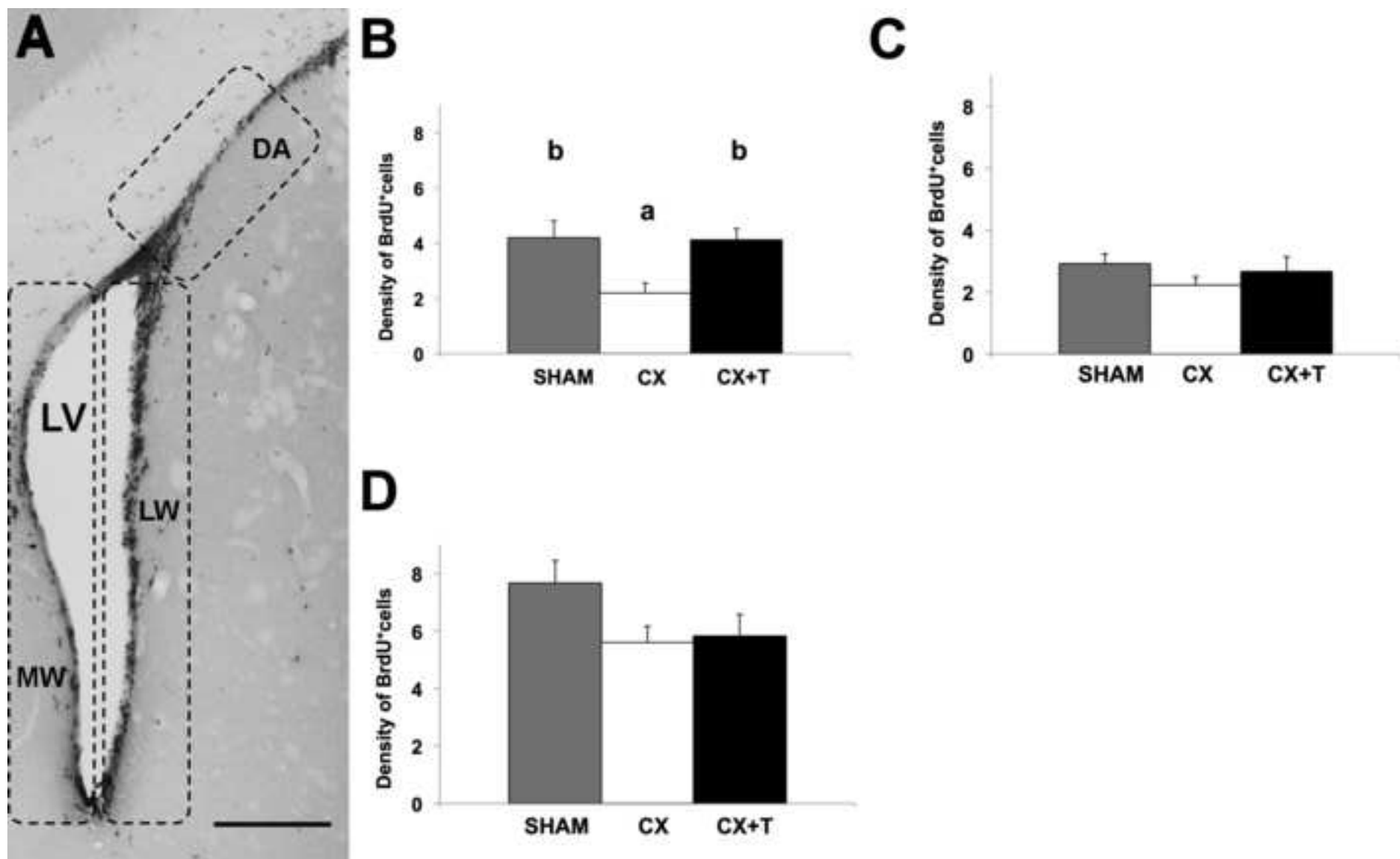
21 **Fig.2** – Variations in BrdU+ cell density in the lateral wall of SVZ of different experimental  
22 groups. A=SHAM, B=CX, C=CX+T, D=CX+E<sub>2</sub>, E=CX+DHT, F=CX+DHT+ E<sub>2</sub>, LV=lateral  
23 ventricle, scale bar= 10  $\mu\text{m}$ . The small box in A shows a high enlargement of two BrdU+  
24 cells, scale bar= 2,5  $\mu\text{m}$ .  
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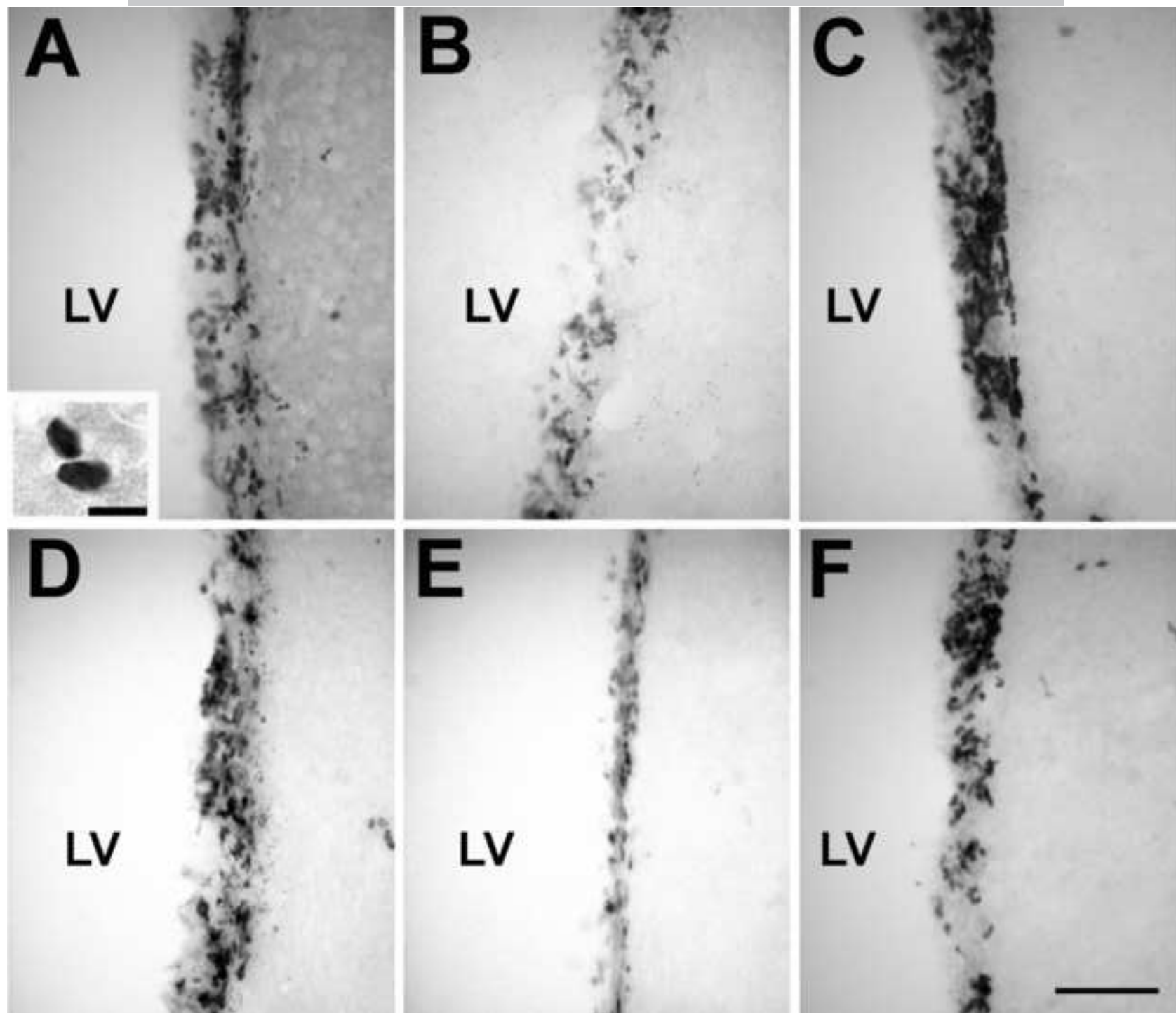
31 **Fig.3** - Histogram illustrating the variations of the density of BrdU labeled cells (Mean $\pm$ SE)  
32 in the lateral wall of the lateral ventricle in the experimental groups of the experiment 2. The  
33 significant differences (ANOVA followed by Tukey test at a level of  $P < 0.05$ ) are denoted by  
34 a or b.  
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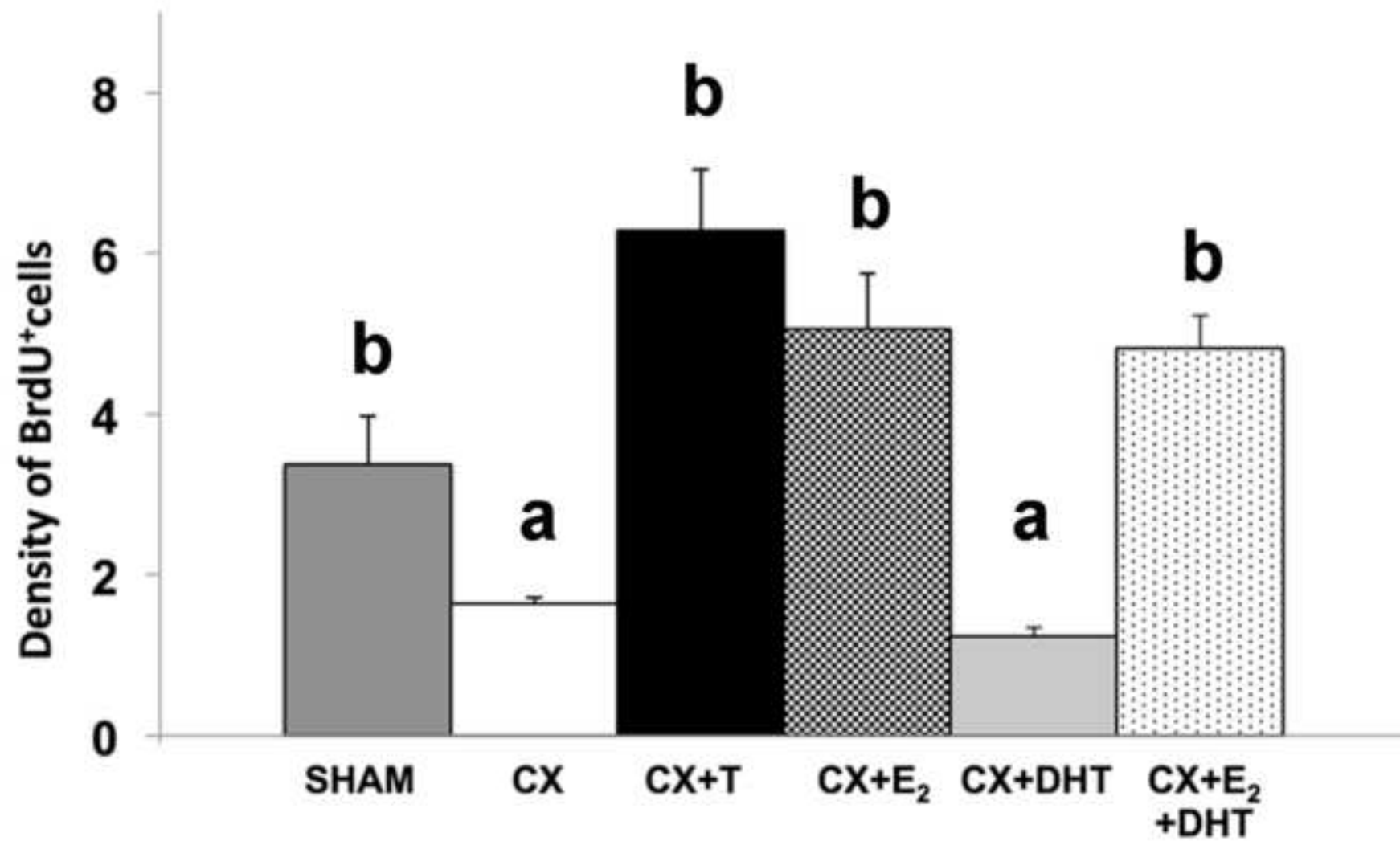
42 **Fig.4** - Histograms illustrating the difference among the experimental groups of the density of  
43 PHH3 (A) and Mash1 (B) positive cells (Mean $\pm$ SE) in the lateral wall of the lateral ventricle.  
44 The significant differences (ANOVA followed by Tukey test at a level of  $P < 0.05$ ) are  
45 denoted by a or b.  
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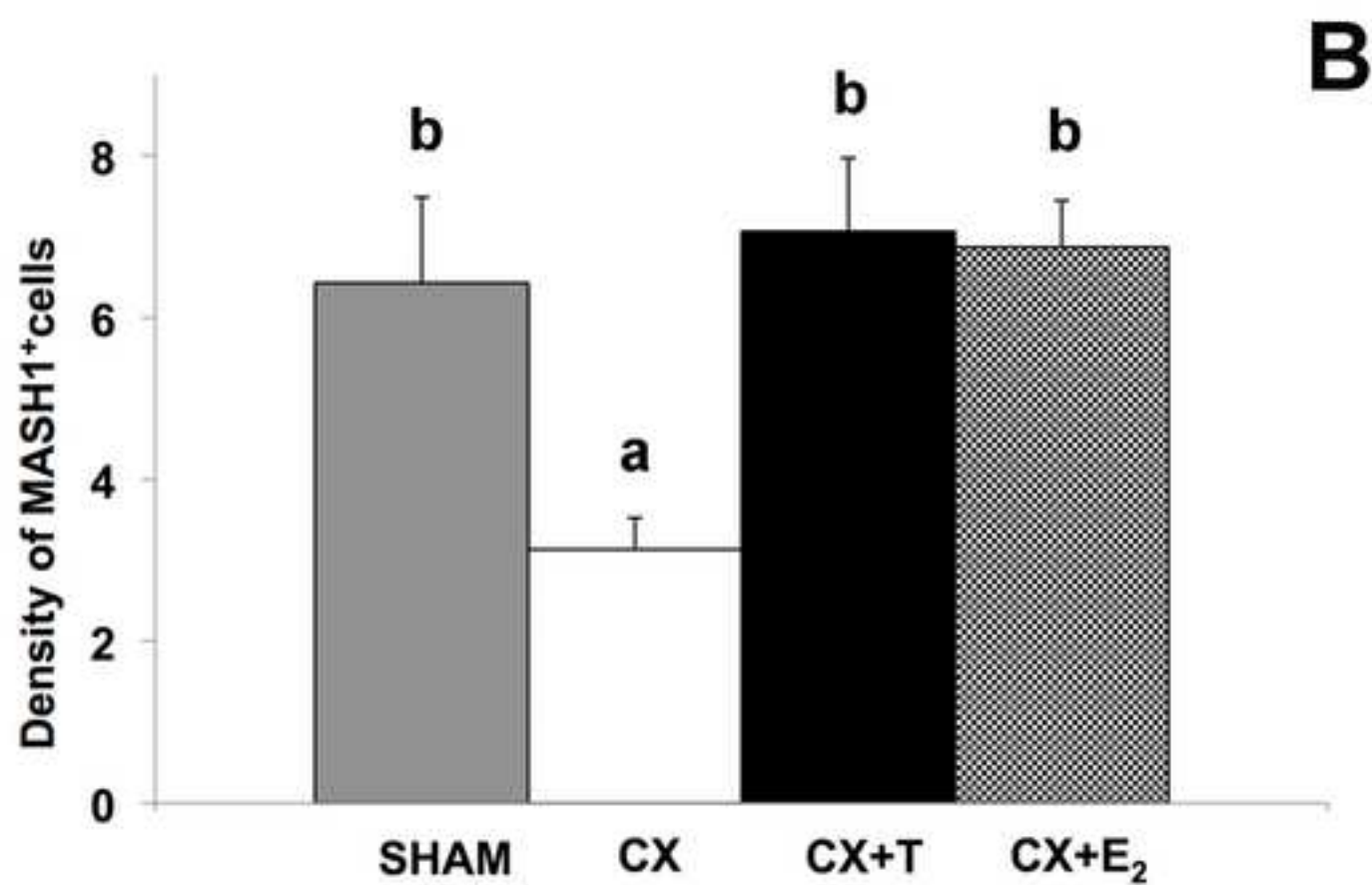
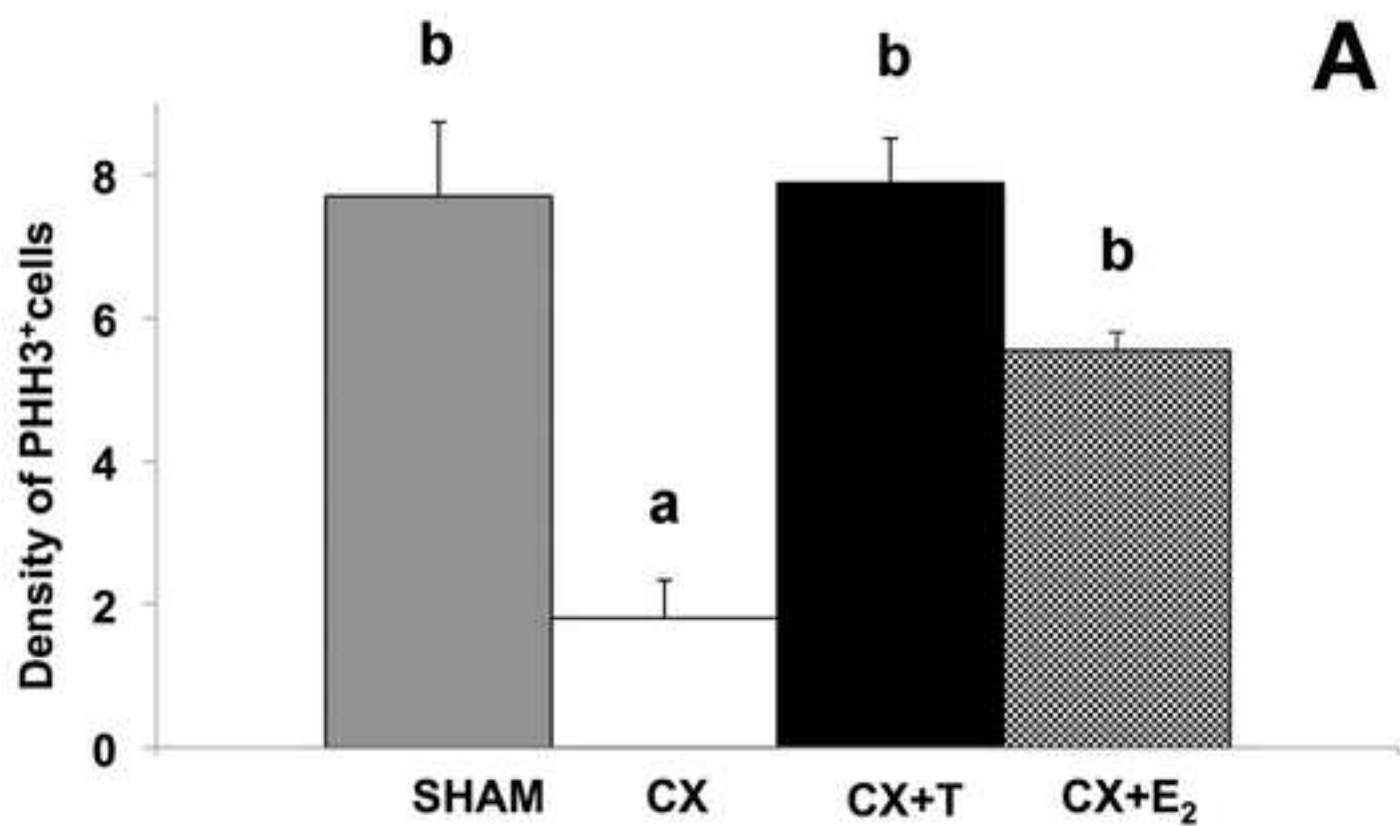
53 **Fig.5** - Density of BrdU+ cells (Mean $\pm$ SE) in the lateral wall of female rats. The  
54 experimental groups did not show statistically significant differences in the density of BrdU  
55 labeled cells.  
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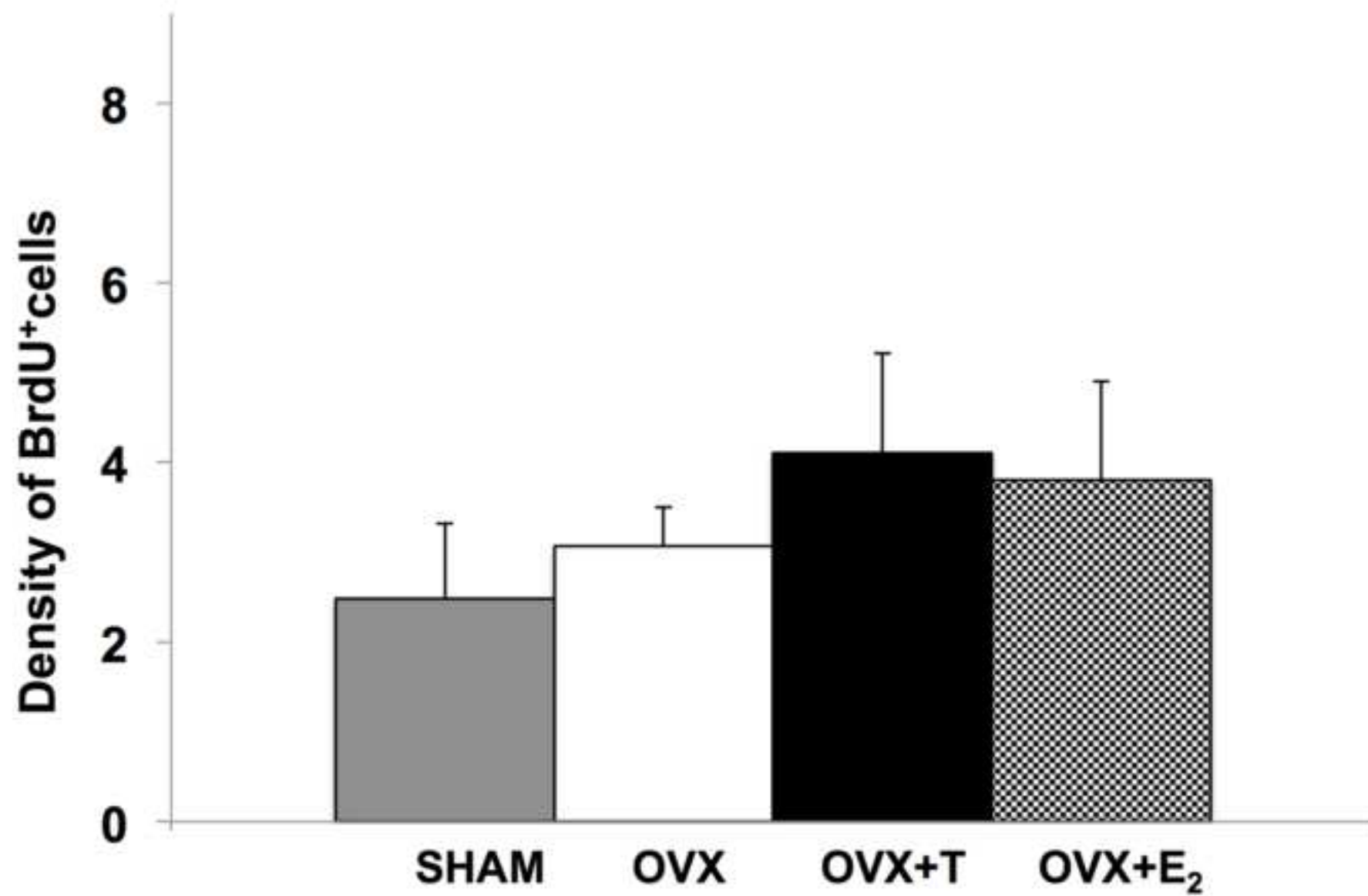












**HIGHLIGHTS**

- Adult gonadectomy reduces cell proliferation in male but not in female rat SVZ
- Testosterone or Estradiol, but not DHT restores cell proliferation only in male rat
- Adult neurogenesis in rats is a process differentially modulated in the two sexes

ACCEPTED MANUSCRIPT

