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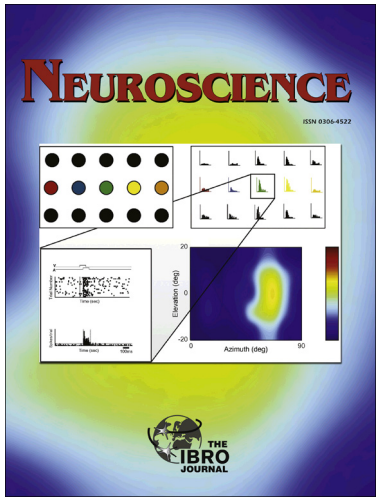

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TESTOSTERONE AND ESTRADIOL DIFFERENTIALLY AFFECT CELL PROLIFERATION IN THE SUBVENTRICULAR ZONE OF YOUNG ADULT GONADECTOMIZED MALE AND FEMALE RATS

SHORT RUNNING TITLE: Sex hormones and proliferation in young adult SVZ

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ABSTRACT

Steroid hormones are important players to regulate adult neurogenesis in the dentate gyrus of hippocampus, but their involvement in the regulation of the same phenomenon in the subventricular zone (SVZ) of the lateral ventricles is not completely understood. Here, in male rats, we tested the existence of activational effects of Testosterone (T) on cell proliferation in the adult SVZ. To this aim, three groups of male rats: castrated, castrated and treated with T, and controls were treated with BrdU and killed after 24 hours. The density of BrdU labeled cells was significantly lower in castrated animals in comparison to the other two groups, thus supporting a direct correlation between SVZ proliferation and levels of circulating T. To clarify whether this effect is purely androgen-dependent, or mediated by the T metabolites, estradiol (E_2) and dihydrotestosterone (DHT), we evaluated SVZ proliferation in castrated males treated with E_2, DHT and E_2+DHT, in comparison to T- and vehicle-treated animals, and sham-operated controls. The stereological analysis demonstrated that E_2 and T, but not DHT, increase proliferation in the SVZ of adult male rats. Quantitative evaluation of cells expressing the endogenous marker of cell proliferation PHH3, or the marker of highly dividing SVZ progenitors Mash1, indicated the effect of T/E_2 is mostly restricted to SVZ proliferating progenitors. The same experimental protocol was repeated on ovariectomized female rats treated with E_2 or T. In this case, no statistically significant difference was found among groups. Overall, our results clearly show that the gonadal hormones T and E_2 represent important mediators of cell proliferation in the adult SVZ. Moreover, we show that such effect is restricted to males, supporting adult neurogenesis in rats is a process differentially modulated in the two sexes.

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

1. INTRODUCTION

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

In the rat DG, gonadal hormones modulate adult neurogenesis in a rather complex way by differentially affecting cell proliferation and survival in males and females (Barker and Galea, 2008). For example, in female rats each phase of the estrous cycle shows a different level of cell proliferation, peaking during the proestrus (when estrogens' levels are highest) and decreasing in estrus or in diestrus (Tanapat et al., 1999). Ovariectomy induces a decrease of cell proliferation, whereas acute treatment with estradiol (E_2) to ovariectomized animals increases the number of newborn cells (Tanapat et al., 2005). By contrast, in male rats,

androgens [testosterone (T) and dihydrotestosterone (DHT)], but not E_2, enhance cell survival in the DG (Spritzer and Galea, 2007).

In the SVZ neurogenic niche such studies have been mostly performed in females by specifically focusing on the effect of E_2 in both naïve and neurodegenerative models (Saravia et al., 2004, Hoyk et al., 2006, Suzuki et al., 2007, Brock et al., 2010, Veyrac and Bakker, 2011). Only indirect evidences indicate that other gonadal hormones, such as progesterone and T can regulate neurogenesis in the adult SVZ niche (Peretto et al., 2001, Giachino et al., 2004, Zhang et al., 2010, Tatar et al., 2013). Similarly to the DG, the modulatory effect of gonadal hormones, particularly of E_2, on SVZ neurogenesis appears highly variable depending on type of treatment/dose (chronic vs. acute), sex, species, and (in mouse) strain considered (Smith et al., 2001, Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker, 2011, Tatar et al., 2013). In addition, such modulation differentially impacts the neurogenic process influencing progenitor cells proliferation and/or integration of newborn neurons in both the main and the accessory OB (Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker, 2011).

Here, for the first time we specifically address the possible impact of T in the regulation of SVZ neurogenesis in the adult rat, focusing on the issue of cell proliferation. In addition, by considering that T in the brain is metabolized into E_2 and DHT (Celotti et al., 1991), we compared the effects exerted by exogenous treatment of acute doses of T and its metabolites in multiple groups of gonadectomized and control animals on both sexes. Our results show that the levels of circulating T and its metabolite E_2, but not DHT, differentially influence cell proliferation in the SVZ of male and female rats. These data further confirm the role of gonadal hormones as important regulative factors of adult SVZ neurogenesis, and definitively demonstrate the involvement of T in such process.

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 20µg/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% isofluorane vaporized in O_2 / N_2O (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

of testis. In males assigned to the sham operated group a small incision was performed in the scrotum and then sutured without removing the testes.

In females assigned to the ovariectomized group both ovaries were removed by two small incisions on the back: the uterus was clamped near the ovary with a silk suture to prevent hemorrhage and each ovary was removed with a single cut of the oviductum near the ovary. In the females of sham-operated group, animals received two small incisions on the back, as in the previous group, but without removing ovaries. Rats (randomly assigned 5 per cage) were then allowed to recovery for two weeks before further treatments.

2.3. Procedure

2.3.1. Hormonal treatments

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

2.3.1.2. Experiment 2: Testosterone metabolites effect in male rat.

Six groups of 42 day-old male rats were used: 1- Castrated + T (CX+T, N = 7); 2- castrated + E_2 (CX+E_2, N = 8); 3- castrated + DHT (CX+DHT, N = 8); 4- castrated + E_2 + DHT (CX+ E_2+DHT, N = 8); 5-castrated + sesame oil (CX+OIL, N = 7); 6- sham operated animals + sesame oil (SHAM, N = 7). All animals received one i.p. injection of hormones or vehicle

(sesame oil) at the following concentrations: T (1.00 mg/0,1 mL sesame oil; Sigma-Aldrich, Milan, Italy); E_2 (β-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_2 (OVX+E_2, N = 5) ((β-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,

cryoprotected in increasing sucrose solutions and frozen in isopentane pre-cooled in dry ice at -30°C / –40°C. Brains were stored at –80°C up to the day before cryosectioning when they were moved to a –20°C freezer. Serial free-floating 40 μm-thick coronal sections were collected in multiwell dishes.

For BrdU immunostaining sections were treated with 2 M HCl for 1 h at 37°C, neutralized with borate buffer pH 8.5, and subsequently incubated overnight with the primary rat anti-BrdU antibody (AbD Serotec, Nottingham, UK, cat. #: OBT0030CX, (Schutte et al., 1987) diluted 1:10,000, with PBS-T (Triton-X100, 0.2% in 0.1 M phosphate buffer saline, PBS) followed by a biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-MC15), diluted 1:250 with PBS-T. Sections were then processed for 1 hour in Avidin-Biotin Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase activity was visualized with a solution containing 0.400 mg/ml of 3,3′-diamino-benzidine (DAB, Sigma, Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6.

For the detection of the phosphorilated form of Histone H3 (PHH3) and Mash1, sections were incubated for 48 h at 4°C with anti-PHH3, 1:4000 (Millipore, Massachusetts, USA, cat. #: 06-570 (Fukushima et al., 2009) and anti-Mash1, 1:2000 (BD Bioscience, New Jersey, USA, cat. #: 556604 (Doetsch et al., 1997), in a solution of PBS, pH 7.4, containing 2% Triton X-100 (Merck, Darmstadt, Germany), 0,5 % Normal Goat Serum (Vector Laboratories, California, USA), and 2% BSA (Sigma-Aldrich, Milan, Italy). Sections were washed and incubated, respectively, with solutions of secondary anti-rabbit 488, 1:400 (Jackson ImmunoResearch Laboratories, Pennsylvania, USA, cat. #: 11-545-144) and with anti-Mouse 647, 1:400 (Jackson ImmunoResearch Laboratories, Pennsylvania, USA, cat. #: 115-605-146). Sections were then cover slipped with antifade mounting medium Mowiol (Sigma-Aldrich, Milan, Italy) and analyzed with a laser scanning Leica TCS SP5 (Leica Microsystems) confocal microscope. Images were processed using Image J

(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 (μm^3), the values are expressed as mean □ Standard Error for 10^4 μm^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, ± Standard Error multiplied respectively, for 10^8 μm^3 (PHH3) and for 10^5 μm^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± 0.366, N=5) in comparison to both control (4.202±0.612, N=5) and CX+T (4.128±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).

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_2, 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_2 and DHT, we treated CX animals with acute doses of T, E_2, DHT, E_2+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±0.620, N=7) was not significantly different ($p>0.05$) from CX+T (6.290±0.752, N=7), CX+E_2 (5.054±0.693, N=8), and CX+DHT+E_2 (4.814±0.405, N=8). By contrast, this number was significantly lower ($p<0.05$) in CX+OIL (1.400±0.054, N=7) and CX+DHT (1.232±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_2, and not to the lack of a pure androgen as DHT.

To investigate the nature of SVZ cell types involved in such T/E_2-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

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

Similarly, the one-way ANOVA for Mash1+ cell density revealed a significant effect of treatment ($F_{(3,8)}=7.023$; $p<0.05$). The post-hoc analysis showed that the density of positive cells in CX+OIL group (3.134±0.395, N=3) was significantly lower ($p<0.05$) than the other three groups (CX+T= 7.071± 0.902, N=3, CX+E2= 6.871± 0.578, N=3, SHAM= 6.761± 0.854, N=3)(Fig. 4B). These data indicate that T/E_2 control the proliferation rate of SVZ progenitors.

3.3. Gonadal hormones T and E_2 do not regulate the SVZ proliferation in prepuberal ovariectomized female rat

In the third experiment we investigated whether, similarly to males, T and/or E_2 may influence the rate of SVZ proliferation also in ovariectomized female rats. Therefore, by using the above reported methodological approach, we performed an additional experiment in females by examining cell proliferation within the SVZ lateral wall in the following groups: ovariectomized (OVX) animals, sham controls (SHAM), OVX females treated with acute doses of T (OVX+T) or with E_2 (OVX+E_2). The density of BrdU+ cells measured through the stereological analysis was not strongly different among the experimental groups (OVX = 3.072±0.425, N=6, OVX+T= 4.101±1.116, N=5, OVX+E2=3.805±1.097, N=5, SHAM=2.479±0.841, N=4). The one-way ANOVA ($F_{(3,16)}= 0.618$, $p>0.05$) confirmed the lack of significant effect of treatments (Fig. 5). Overall, these results indicate that in contrast to males, the acute treatment with T or E_2 does not influence the proliferation in the SVZ of females ovariectomized in prepuberal period and show the occurrence of a novel sex dimorphism related to the regulation of cell proliferation in the SVZ region of rats.

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_2 and not by the androgenic metabolite DHT. By contrast, we demonstrate that, in the same experimental conditions, T or E_2 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_2 treatment in prepuberal castrated animals increases both the PHH3 proliferating cells and the Mash1 positive Type C cells.

Interestingly, the action of T/E_2 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_2 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

few studies are at the moment available concerning the impact of gonadal hormones on OB neurogenesis (Smith et al., 2001, Hoyk et al., 2006, Brock et al., 2010, Veyrac and Bakker, 2011), despite their contribution to olfactory reproductive behavior (Keller et al., 2009), and recent data supporting a link between OB neurogenesis and reproduction (reviewed in Peretto and Paredes, 2014).

We found that two weeks after gonadectomy, a condition that virtually implies absence of significant levels of circulating sex hormones (Kashiwagi et al., 2005), the amount of cell proliferation in the SVZ, evaluated 24 hours after the BrdU treatment, was significantly reduced in comparison to intact or sham-operated animals. Moreover, we found that a single injection of T restores in short time (24 hours) the level of cell proliferation. To date, this is the first direct evidence demonstrating that in male rats the lack of T affects adult neurogenesis in the SVZ, by reducing the rate of cell proliferation.

It has been reported that gonadectomy affects adult neurogenesis also in mice, but with an opposite effect, that is, increasing cell proliferation in SVZ, however these effects are strain- and sex-dependent (Tatar et al., 2013). Finally, in the Sprague Dawley rat, androgens enhance adult neurogenesis in DG, promoting cell survival rather than proliferation (Spritzer and Galea, 2007, Hamson et al., 2013). The results of the first experiment prompted us to investigate whether T-dependent proliferation in male rat SVZ was mediated by either or both T metabolites: E_2 and DHT. Indeed, in the brain both metabolites are present due to the action of the enzymes aromatase (converting T into E_2) and 5α-reductase (converting T into DHT) (Celotti et al., 1991).

We found that acute administration of E_2 but not DHT was effective in restoring SVZ proliferation in castrated males. This result indicates that the proliferative activity elicited by T in the SVZ is mediated by its conversion in E_2. In contrast, in the rat DG, chronic administration of T or DHT, but not of E_2, was found to modulate hippocampal neurogenesis

(Spritzer and Galea, 2007). However, such effect was due to enhanced cell survival rather than proliferation. Thus, since our study was restricted to only short-survival times after BrdU administration (i.e., proliferation), further analyses are necessary to establish whether diverse gonadal hormones (e.g., DHT) differentially affect the two niches, and/or specific steps of the SVZ neurogenic process (i.e., migration and differentiation).

Estrogens may exert protective effects under pathological conditions in several brain regions (Garcia-Segura et al., 2001, Behl, 2002) including adult neurogenic regions. An estrogen-dependent modulation of DG and SVZ neurogenesis in males was found in a mice model of induced diabetes. In these animals, E_2 restores the rate of SVZ proliferation in streptozotocin-diabetic male mice, contrasting the reduction of cell proliferation observed in non-E_2 treated males (Saravia et al., 2004). However, E_2 is not inducing any increase in control animals, thus supporting the hypothesis that, in male mice, E_2 can stimulate SVZ proliferation only in pathological conditions.

Overall, these results underline the importance of carefully consider the species, the region and type/nature of progenitors and the experimental condition, including the age at the time of gonadectomy as well as that at the time of hormonal treatments, when studying the effect of such hormones in the regulation of the two adult neurogenic niches (Galea et al., 2013).

Interestingly, in our study, the SVZ population shows a sexually dimorphic sensitivity to gonadal hormones. In fact, we found that both ovariectomy and acute administration of T or E_2 in OVX females did not modify the number of cycling cells in the SVZ neurogenic niche. This sex difference is not limited to SVZ: in the DG, a sex-dependent regulation of adult neurogenesis has been already described in different rodent species (for a recent review see (Galea et al., 2013).

The way by which E_2 interacts with SVZ in female is dependent on the species, dose- and time of administration. For example, in the praire vole, estrous induction, which in this

species is driven by exposure to unfamiliar male chemosensory cues (Richmond and Conaway, 1969), is associated to an estrogen-dependent increase of SVZ cell proliferation (Smith et al., 2001). By contrast, in C57Bl/6j/sv129 female mice chronically exposed to E_2, newborn cell survival in the OB decreases and SVZ cell proliferation is not modified (Veyrac and Bakker, 2011). On the contrary, a short-term exposure to E_2 negatively affects cell proliferation in C57Bl6/J female mice SVZ (Brock et al., 2010). Interestingly, in OVX female mice of the same strain, a chronic treatment with low doses of E_2 stimulates cell proliferation in the SVZ of post-stroke animals, although such increase was not present in control animals (Suzuki et al., 2007). In rats, a chronic treatment with E_2 on OVX females results in a minor number of newborn neurons integrating in the accessory OB (Hoyk et al., 2006), however, in this study neurogenesis was evaluated only at long survival time after BrdU injection, thus it is not clear if this is an effect on cell proliferation or on cell survival. Our data indicate that prepuberal ovariectomy, as well as short-term exposure to E_2, does not affect the SVZ proliferation in female rat.

However, estrogen receptors are expressed in vivo and in vitro in cells originated, or located in the rat SVZ lining the lateral ventricles (Brannvall et al., 2005, Isgor and Watson, 2005), thus long term effects of E_2 in females (e.g., increasing the responsiveness of newborn neurons during their survival/differentiation in the OB) cannot be ruled out.

In conclusion, we think that our results, although not definitive, add a small but important tile in the complex emerging picture describing the impact of gonadal hormones on adult neurogenesis. Future studies, besides filling the still existing gaps of knowledge related to this specific issue (e.g., expression analysis of gonadal hormone receptors in different SVZ neurogenic niche cell types in males and females; role of T and or E_2 in mediating integration/survival of newborn neurons in both sexes) should be directed to unravel whether a reciprocal interplay exists between gonadal hormones, adult neurogenesis, and other

hypothalamic neuroendocrine signals/nuclei involved in the control the gonadal hormones secretion.

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6. REFERENCES

Abrous DN, Koehl M, Le Moal M (2005). Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85:523-569.

Barker JM, Galea LA (2008). Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats. Neuroscience 152:888-902.

Behl C (2002). Oestrogen as a neuroprotective hormone. Nat Rev Neurosci 3:433-442.

Bonfanti L, Peretto P (2011). Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci 34:930-950.

Brannvall K, Bogdanovic N, Korhonen L, Lindholm D (2005). 19-Nortestosterone influences neural stem cell proliferation and neurogenesis in the rat brain. Eur J Neurosci 21:871-878.

Brock O, Keller M, Veyrac A, Douhard Q, Bakker J (2010). Short term treatment with estradiol decreases the rate of newly generated cells in the subventricular zone and main olfactory bulb of adult female mice. Neuroscience 166:368-376.

Celotti F, Melcangi RC, Negri-Cesi P, Poletti A (1991). Testosterone Metabolism in Brain Cells and Membranes. J Steroid Biochem Mol Biol 40:673-678.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046-5061.

Feierstein CE (2012). Linking adult olfactory neurogenesis to social behavior. Front Neurosci 6:173.

Fukushima S, Terasaki M, Sakata K, Miyagi N, Kato S, Sugita Y, Shigemori M (2009). Sensitivity and usefulness of anti-phosphohistone-H3 antibody immunostaining for counting mitotic figures in meningioma cases. Brain Tumor Pathol 26:51-57.

Galea LA (2008). Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. Brain Res Rev 57:332-341.

Galea LA, Wainwright SR, Roes MM, Duarte-Guterman P, Chow C, Hamson DK (2013). Sex, hormones, and neurogenesis in the hippocampus: Hormonal modulation of neurogenesis and potential functional implications. J Neuroendocrinol 25:1039-1061.

Garcia-Segura LM, Azcoitia I, DonCarlos LL (2001). Neuroprotection by estradiol. Prog Neurobiol 63:29-60.

Giachino C, Galbiati M, Fasolo A, Peretto P, Melcangi RC (2004). Effects of progesterone derivatives, dihydroprogesterone and tetrahydroprogesterone, on the subependymal layer of the adult rat. J Neurobiol 58:493-502.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al. (1988). Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379-394.

Gundersen HJ, Jensen EB, Kieu K, Nielsen J (1999). The efficiency of systematic sampling in stereology--reconsidered. J Microsc 193:199-211.

Hamson DK, Wainwright SR, Taylor JR, Jones BA, Watson NV, Galea LA (2013). Androgens increase survival of adult-born neurons in the dentate gyrus by an androgen receptor-dependent mechanism in male rats. Endocrinology 154:3294-3304.

Hoyk Z, Varga C, P√°rducz A (2006). Estrogen-induced region specific decrease in the density of 5-bromo-2-deoxyuridine-labeled cells in the olfactory bulb of adult female rats. Neuroscience 141:1919-1924.

Isgor C, Watson SJ (2005). Estrogen receptor alpha and beta mRNA expressions by proliferating and differentiating cells in the adult rat dentate gyrus and subventricular zone. Neuroscience 134:847-856.

Kashiwagi B, Shibata Y, Ono Y, Suzuki R, Honma S, Suzuki K (2005). Changes in testosterone and dihydrotestosterone levels in male rat accessory sex organs, serum, and seminal fluid after castration: establishment of a new highly sensitive simultaneous androgen measurement method. J Androl 26:586-591.

Keller M, Baum MJ, Brock O, Brennan PA, Bakker J (2009). The main and the accessory olfactory systems interact in the control of mate recognition and sexual behavior. Behav Brain Res 200:268-276.

Larsen C, Grattan D (2012). Prolactin, neurogenesis, and maternal behaviors. Brain, Behav, Immun 26:201-209.

Lennington JB, Yang Z, Conover JC (2003). Neural stem cells and the regulation of adult neurogenesis. Reprod Biol Endocrinol 1:99.

Merkle FT, Fuentealba LC, Sanders TA, Magno L, Kessaris N, Alvarez-Buylla A (2014). Adult neural stem cells in distinct microdomains generate previously unknown interneuron types. Nat Neurosci 17:207-214.

Merkle FT, Mirzadeh Z, Alvarez-Buylla A (2007). Mosaic organization of neural stem cells in the adult brain. Science 317:381-384.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. San Diego, USA: Academic Press.

Peretto P, Giachino C, Panzica GC, Fasolo A (2001). Sexually dimorphic neurogenesis is topographically matched with the anterior accessory olfactory bulb of the adult rat. Cell Tissue Res 306:385-389.

Peretto P, Paredes RG (2014) Social Cues, Adult Neurogenesis, and Reproductive Behavior. In: Neurobiology of Chemical Communication(Mucignat-Caretta, C., ed) Boca Raton (FL).

Ponti G, Obernier K, Guinto C, Jose L, Bonfanti L, Alvarez-Buylla A (2013). Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. Proc Natl Acad Sci U S A 110:E1045-1054.

Richmond M, Conaway CH (1969). Management, breeding, and reproductive performance of the vole, Microtus ochrogaster, in a laboratory colony. Lab Anim Care 19:80-87.

Sahay A, Wilson DA, Hen R (2011). Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. Neuron 70:582-588.

Saravia F, Revsin Y, Lux-Lantos V, Beauquis J, Homo-Delarche F, De Nicola AF (2004). Oestradiol restores cell proliferation in dentate gyrus and subventricular zone of streptozotocin-diabetic mice. J Neuroendocrinol 16:704-710.

Schutte B, Reynders MM, Bosman FT, Blijham GH (1987). Studies with anti-bromodeoxyuridine antibodies: II. Simultaneous immunocytochemical detection of antigen expression and DNA synthesis by in vivo labeling of mouse intestinal mucosa. J Histochem Cytochem 35:371-374.

Smith M, Pencea V, Wang Z, Luskin M, Insel T (2001). Increased number of BrdU-labeled neurons in the rostral migratory stream of the estrous prairie vole. Horm Behav 39:11-21.

Spritzer MD, Galea LA (2007). Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. Dev Neurobiol 67:1321-1333.

Spritzer MD, Ibler E, Inglis W, Curtis MG (2011). Testosterone and social isolation influence adult neurogenesis in the dentate gyrus of male rats. Neuroscience 195:180-190.

Suzuki S, Gerhold LM, Bottner M, Rau SW, Dela Cruz C, Yang E, Zhu H, Yu J, Cashion AB, Kindy MS, Merchenthaler I, Gage FH, Wise PM (2007). Estradiol enhances neurogenesis following ischemic stroke through estrogen receptors alpha and beta. J Comp Neurol 500:1064-1075.

Tanapat P, Hastings NB, Gould E (2005). Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J Comp Neurol 481:252-265.

Tanapat P, Hastings NB, Reeves AJ, Gould E (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci 19:5792-5801.

Tatar C, Bessert D, Tse H, Skoff RP (2013). Determinants of central nervous system adult neurogenesis are sex, hormones, mouse strain, age, and brain region. Glia 61:192-209.

Tong CK, Alvarez-Buylla A (2014). SnapShot: adult neurogenesis in the V-SVZ. Neuron 81:220-220 e221.

Vadodaria KC, Gage FH (2014). SnapShot: adult hippocampal neurogenesis. Cell 156:1114-1114 e1111.

Vetter-O'Hagen CS, Spear LP (2012). Hormonal and physical markers of puberty and their relationship to adolescent-typical novelty-directed behavior. Dev Psychobiol 54:523-535.

Veyrac A, Bakker J (2011). Postnatal and adult exposure to estradiol differentially influences adult neurogenesis in the main and accessory olfactory bulb of female mice. FASEB 25:1048-1057.

Zhang Z, Yang R, Zhou R, Li L, Sokabe M, Chen L (2010). Progesterone promotes the survival of newborn neurons in the dentate gyrus of adult male mice. Hippocampus 20:402-412.

Zhao C, Deng W, Gage FH (2008). Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660.

Legend to the figures

Fig.1 - Variations in the density of BrdU+ cells in the SVZ of castrated males treated with testosterone. **A.** Representative section of the SVZ from a sham-operated male. The rectangles show the 3 regions that were measured in Experiment 1. LV=lateral ventricle, DA=dorsal area, MW=medial wall, LW=lateral wall, scale bar=300 μm. **B-C.** Histograms illustrating the variations of the total number of BrdU labeled cells (Mean±SE) in the lateral wall (B), in the medial wall (C) and in the dorsal area (D). The significant differences (ANOVA followed by Tukey test at a level of $P < 0.05$) are denoted by a or b.

Fig.2 – Variations in BrdU+ cell density in the lateral wall of SVZ of different experimental groups. A=SHAM, B=CX, C=CX+T, D=CX+E_2, E=CX+DHT, F=CX+DHT+ E_2, LV=lateral ventricle, scale bar= 10 μm. The small box in A shows a high enlargement of two BrdU+ cells, scale bar= 2,5 μm.

Fig.3 - Histogram illustrating the variations of the density of BrdU labeled cells (Mean±SE) in the lateral wall of the lateral ventricle in the experimental groups of the experiment 2. The significant differences (ANOVA followed by Tukey test at a level of $P < 0.05$) are denoted by a or b.

Fig.4 - Histograms illustrating the difference among the experimental groups of the density of PHH3 (A) and Mash1 (B) positive cells (Mean±SE) in the lateral wall of the lateral ventricle. The significant differences (ANOVA followed by Tukey test at a level of $P < 0.05$) are denoted by a or b.

Fig.5 - Density of BrdU+ cells (Mean±SE) in the lateral wall of female rats. The experimental groups did not show statistically significant differences in the density of BrdU labeled cells.

Figure 1

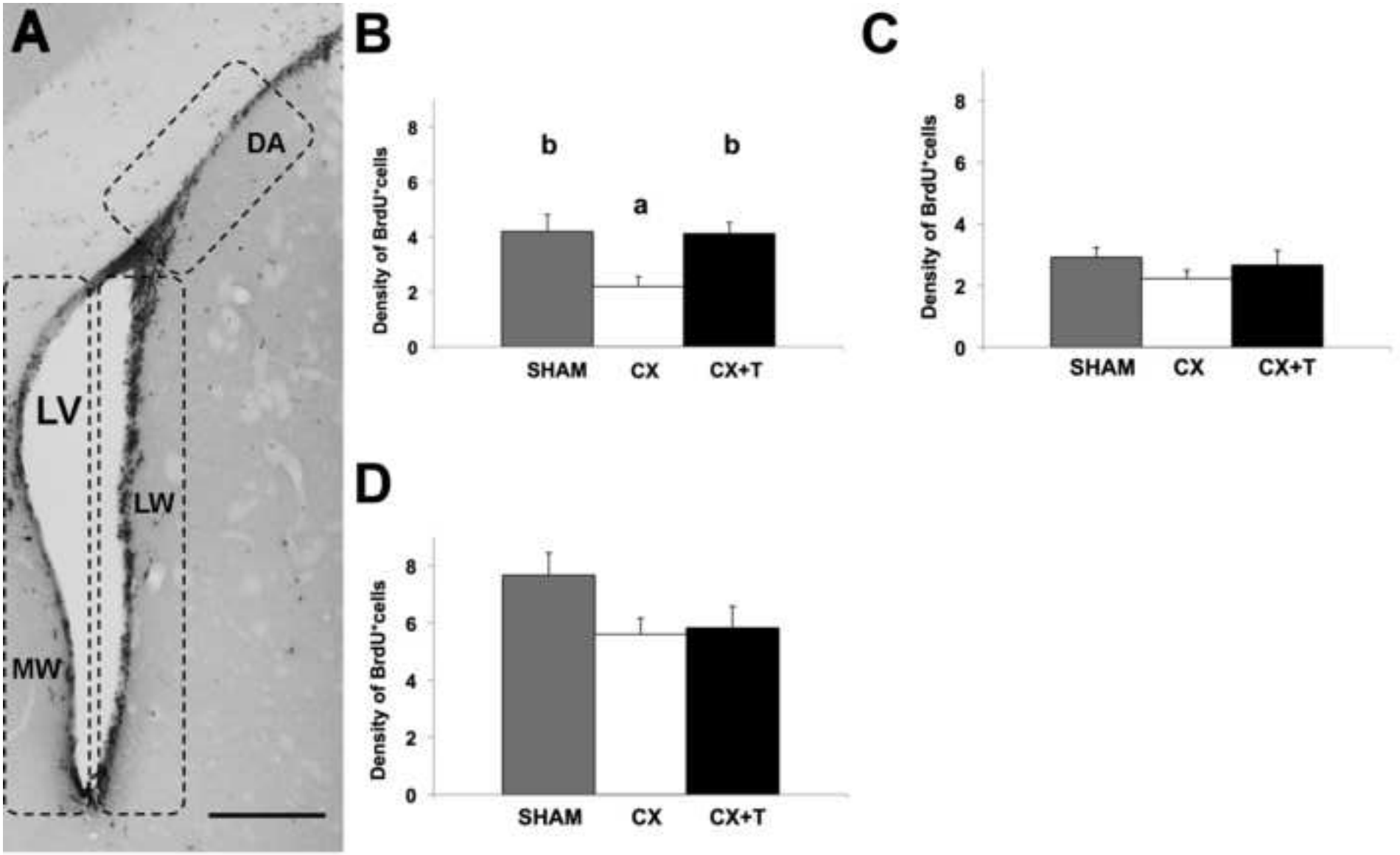


Figure 2

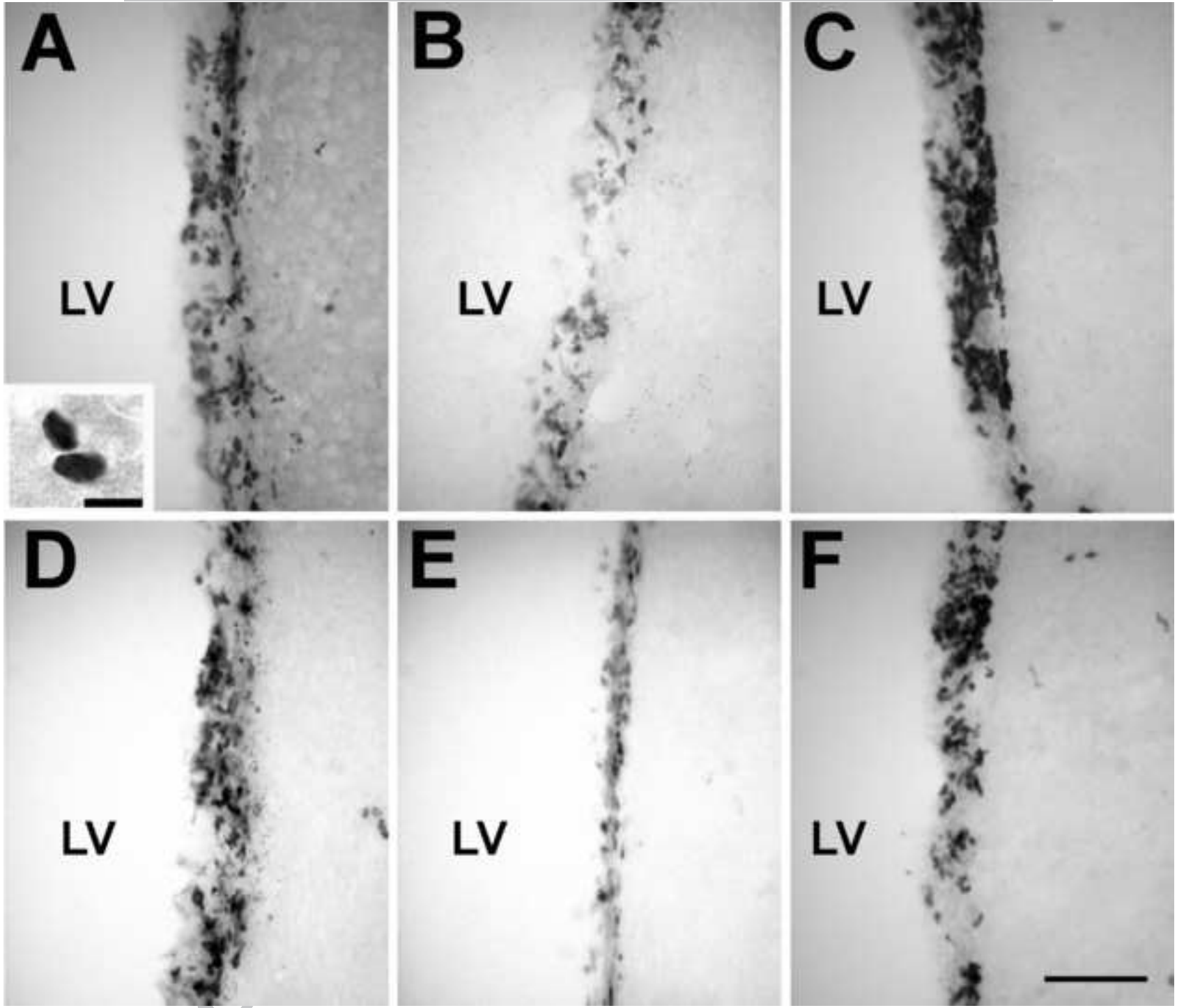


Figure 3

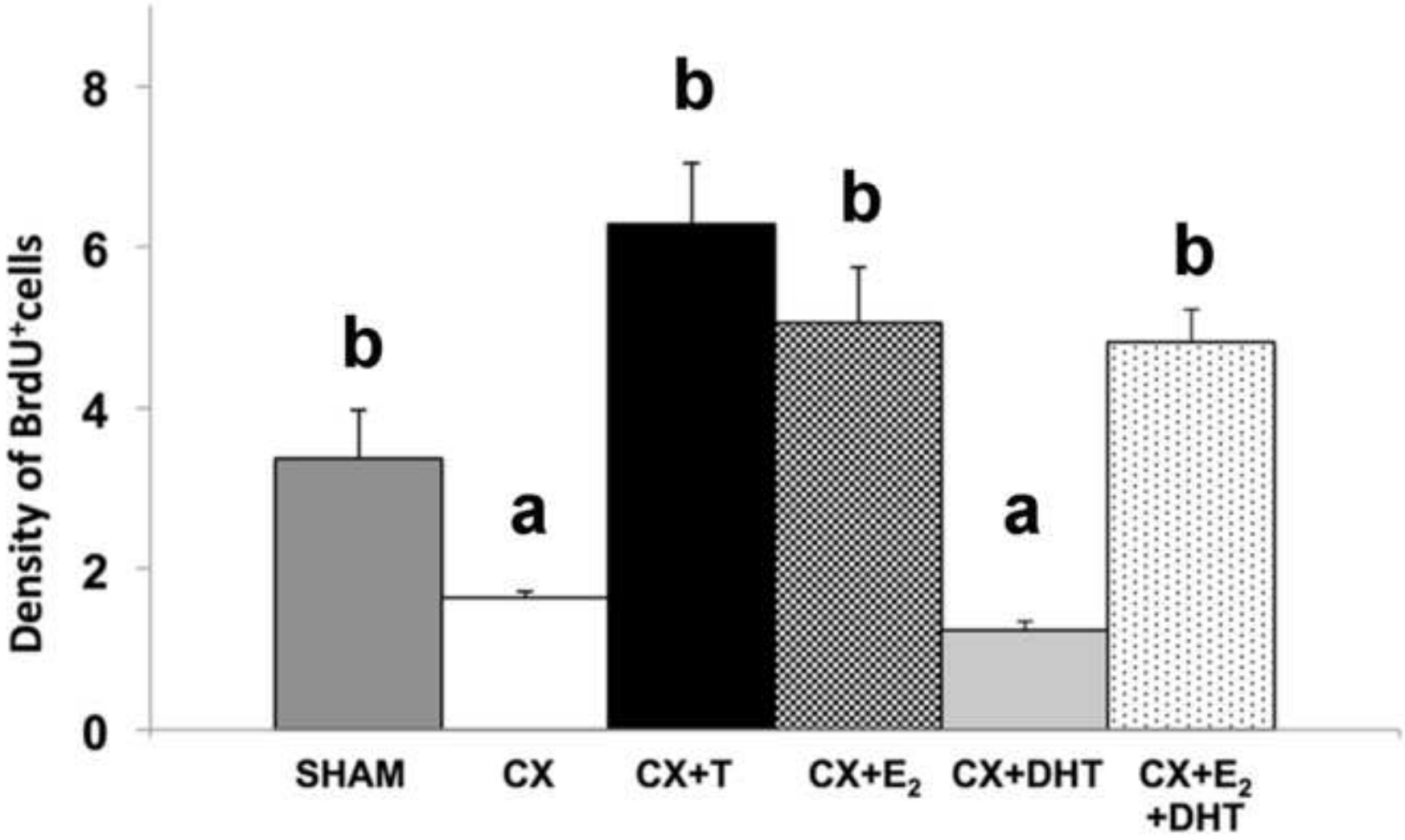


Figure 4

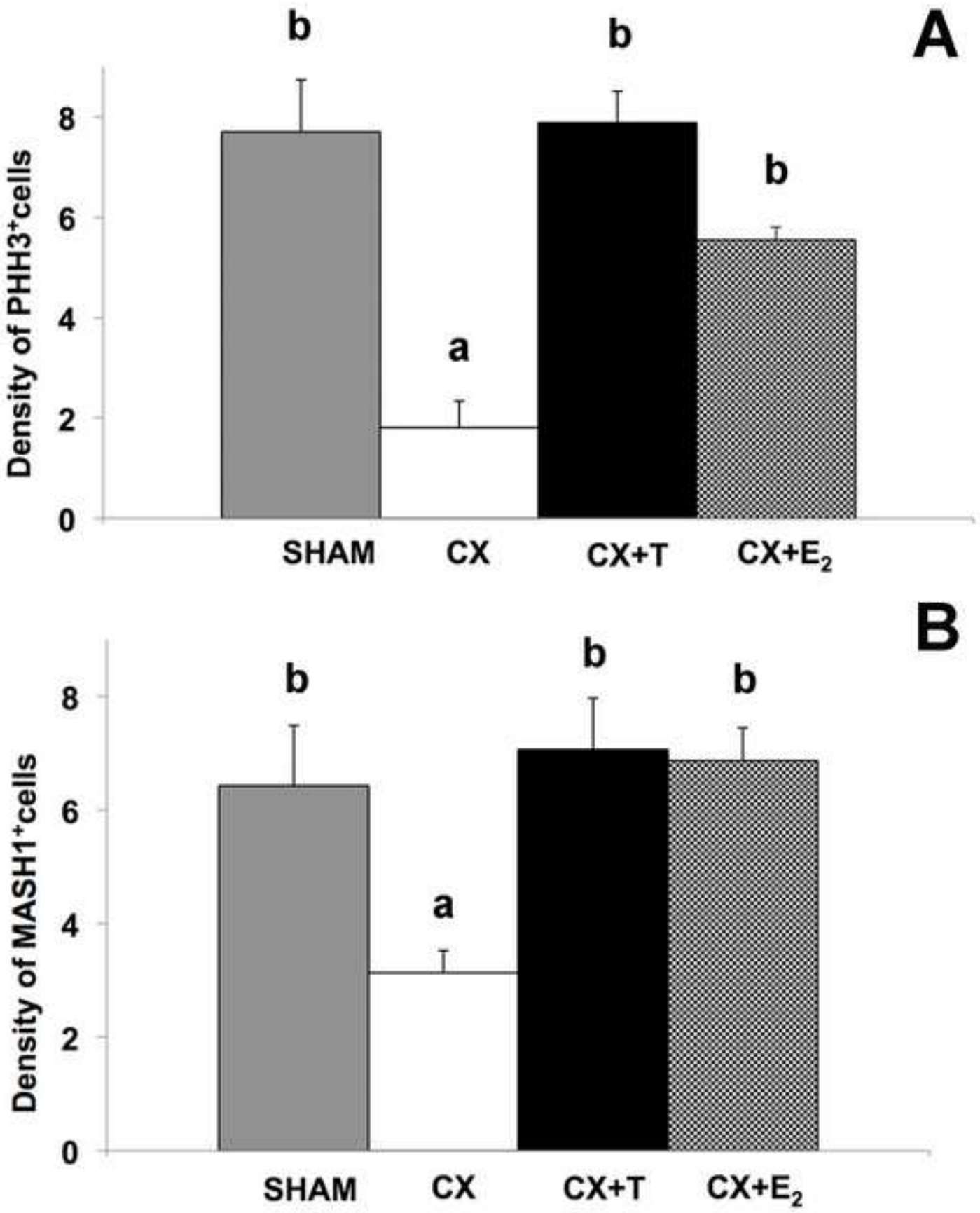
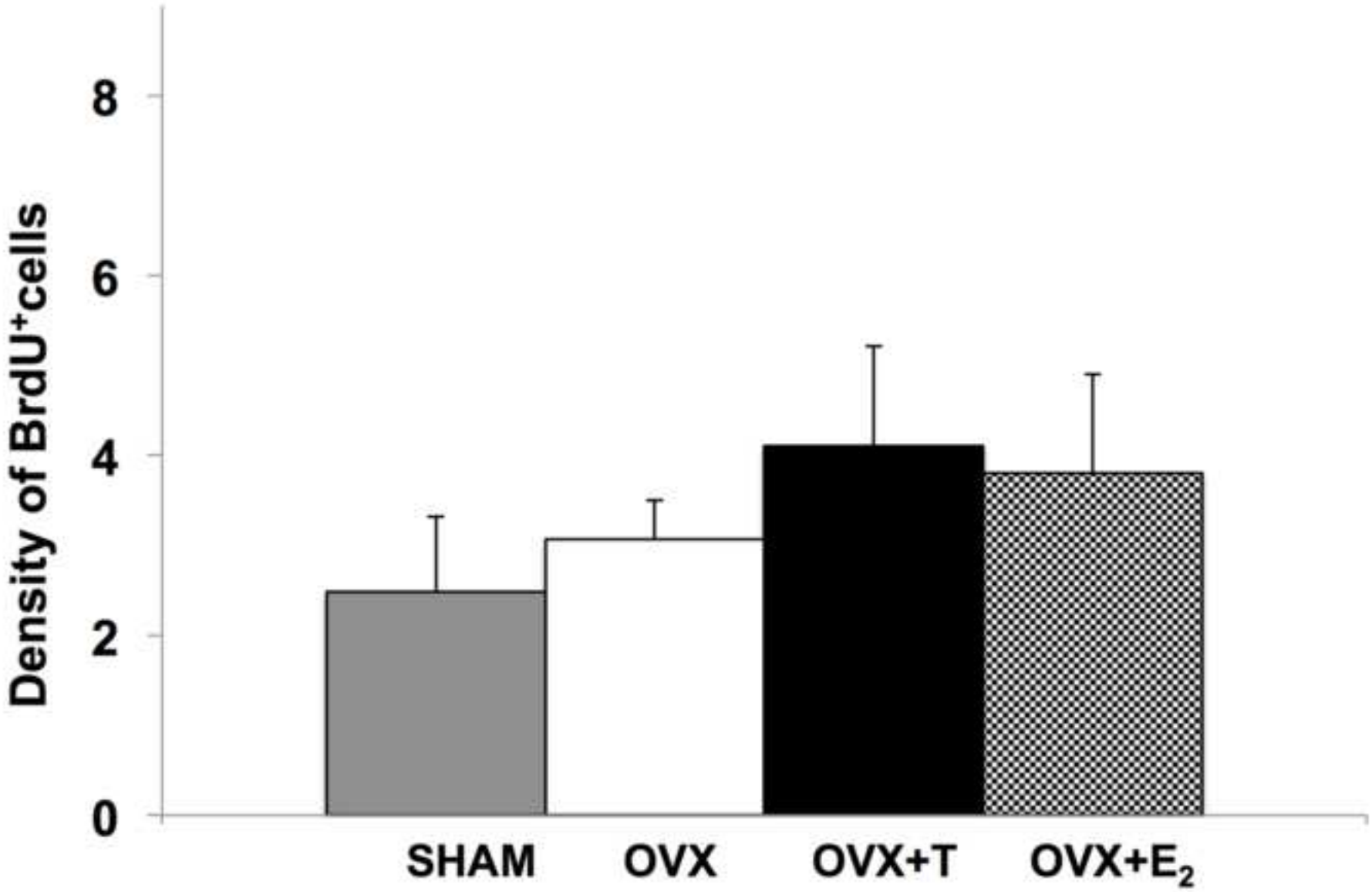


Figure 5



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