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MATERNAL ADMINISTRATION OF FLUTAMIDE DURING LATE GESTATION AFFECTS THE BRAIN AND REPRODUCTIVE ORGANS DEVELOPMENT IN THE RAT MALE OFFSPRING

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Abstract—We have previously demonstrated that male rats exposed to stress during the last week of gestation present age-specific impairments of brain development. Since the organization of the fetal developing brain is subject to androgen exposure and prenatal stress was reported to disrupt perinatal testosterone surges, the aim of this research was to explore whether abnormal androgen concentrations during late gestation affects the morphology of the prefrontal cortex (PFC), hippocampus (HPC) and ventral tegmental area (VTA), three major areas that were shown to be affected by prenatal stress in our previous studies. We administered 10-mg/kg/day of the androgen receptor antagonist *flutamide* (4′nitro-3′-trifluoromethylsobutyranilide) or vehicle injections to pregnant rats from days 15–21 of gestation. The antiandrogenic effects of flutamide were confirmed by the analysis of androgen-dependent developmental markers: flutamide-exposed rats showed reduced anogenital distance, delay in the completion of testis descent, hypospadias, cryptorchidism and atrophied seminal vesicles. Brain morphological studies revealed that prenatal flutamide decreased the number of MAP2 (a microtubule-associated protein type 2, present almost exclusively in dendrites) immunoreactive neuronal processes in all evaluated brain areas, both in prepubertal and adult offspring, suggesting that prenatal androgen disruption induces long-term reductions of the dendritic arborization of several brain structures, affecting the normal connectivity between areas. Moreover, the number of tyrosine hydroxylase (TH)-immunopositive neurons in the VTA of prepubertal offspring was reduced in flutamide rats but reach normal values at adulthood. Our results demonstrate that the effects of prenatal flutamide on the offspring brain morphology resemble several prenatal stress effects suggesting that the mechanism of action of prenatal stress might be related to the impairment of the organizational role of androgens on brain development.

Key words: prenatal flutamide, male rat offspring, prefrontal cortex, hippocampus, ventral tegmental area, tyrosine hydroxylase.

INTRODUCTION

An efficient establishment of synaptic circuits during maturation is essential for the development of normal brain function. The majority of excitatory synapses are formed on dendritic spines and changes in spine density and morphology account for functional differences at the synaptic level (Segal, 2010). The cerebral cortex and the hippocampal formation are essential components of the neural pathways that mediate stress responses and are essential for learning and memory formation (Madeira and Lieberman, 1995). On the other hand the *mesocorticolimbic DA (dopaminergic) system*, that comprises neurons from the ventral tegmental area (VTA) projecting mainly to the hippocampus (HPC) and the prefrontal cortex (PFC) (Kuhar et al., 1999; Chinta and Andersen, 2005; Baier et al., 2012), regulates diverse behavioral and cognitive functions that are crucial for the integration of individual perception and its adaptation to the environment (Missale et al., 1998). During the last years, increasing evidences from rodent models demonstrate that exposure to different stressful events during the last week of gestation strongly impacts on structural and functional fetal central nervous system development, leading to impaired adaptation to stressful conditions, enhanced propensity to self-administer drugs, vulnerability to anxiety and learning deficits (Darnaudery and Maccari,

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Abbreviations: AcB, acetate buffer; ANOVA, analysis of variance; AR, androgen receptor; DA, dopaminergic; DHT, dihydrotestosterone; FLU, flutamide (4′nitro-3′-trifluoromethylsobutyranilide); GD, gestational days; HPC, hippocampus; MAP2, microtubule-associated protein type 2; NeuN, neuron-specific nuclear antigen; PBS, phosphate-buffered saline; PFC, prefrontal frontal cortex; PND, postnatal day; TH, tyrosine hydroxylase; VEH, vehicle; VTA, ventral tegmental área.

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2008; Huizink et al., 2004; Weinstock, 2001, 2008). In addition, the offspring display anomalies in neuronal development and brain morphology which persist into adulthood (Fride and Weinstock, 1989). Our laboratory has a long-standing interest in the effects of prenatal stress on the brain development, especially on the mesocorticolimbic DA pathway (Baier et al., 2012). We have demonstrated that several impairments induced by prenatal stress on the DA metabolism were differentially affected if assayed before or after puberty. This observation confirms the suggestion from previous investigations that perinatal events might render the DA circuitry more vulnerable to puberty variation of the hormonal circulating levels (Diaz et al., 1997). However, the reduction in dendritic arborizations induced by prenatal stress in PFC and HPC, that were reported to occur at adult stages (Barros et al., 2006), were also found prepubertally (Pallares et al., 2013b), suggesting that some plastic morphological processes might be programed prenatally but are relatively insensitive to the increase of sexual hormones during puberty.

The effect of gonadal hormones on brain maturation takes place at two different periods of life known as the classical *organizational/activational* hypothesis of gonadal steroid action (Alonso and Lopez-Coviella, 1998). During the prenatal period, gonadal steroid hormones (i.e. estrogens and androgens) *organize* the developing brain by changing the architecture of several neural substrates which later in puberty are *activated* by the gonadal steroids surge in a directed manner. The direction of adult hormonal responsiveness will dictate sex-specific behavior and physiology (Zhang et al., 2010). In males, testosterone and its 5-alpha reduced metabolite dihydrotestosterone (DHT) are the major circulating androgenic hormones. In rats, androgens-induced masculinization of the reproductive tract and brain sexual behavior takes place over a limited period of perinatal development called *the critical period of differentiation* which is initiated with testosterone peaks on gestational days (GD) 18–19 and is extended up to the first postnatal week (Corbier et al., 1978; Lee et al., 1975). During this period, androgen-dependent tissues are intensely modified (Knickmeyer and Baron-Cohen, 2006). However, the occurrence of some factors during this perinatal phase can interfere with the physiological, morphological, behavioral, and neuroanatomical differences between males and females (Scott et al., 2009). For example, it was reported that prenatal stress suppresses the surge of prenatal testosterone, affecting the male reproductive tract formation, inducing abnormal testosterone levels and feminizing the male sexual behavior (Barros et al., 2004; Gerardin et al., 2005; Shono and Suita, 2003). In our hands, we have shown that prenatal stress induced long-term imbalance of male sexual hormons concentrations in serum, advanced the spermatogenesis development and exerted an age-dependent misbalance on alpha receptor expression on PFC and HPC brain areas (Pallares et al., 2013a,b). Moreover, it was observed that physiological and behavioral damage caused by prenatal stress was prevented by replacement with neonatal testosterone (Pereira et al., 2006), corroborating the importance of neonatal testosterone surge during the sexual differentiation process of the brain. The fetal rat brain expresses androgen receptors (ARs) as early as GD 12 with a peak expression at GD 17–18 (Brannvall et al., 2005). The majority of studies examining the effects of early gonadal action on the adult male rat have focused on sexual behaviors or anatomical aspects of sexually dimorphic central nucleus. In contrast, the effects of early-life manipulation of gonadal steroids on the development of other brain regions outside the hypothalamus were poorly explored.

Androgen organizational influence over mesostriatal and mesolimbic DA system was demonstrated by Creutz and Kritzer (2004). Moreover, Yang and Shieh (2007) suggested that gonadal hormones play a regulatory role in the stimulation of cocaine and amphetamine-regulated transcript peptide in mesolimbic and nigrostriatal DA system and Johnson et al. (2010) demonstrated that testosterone play a suppressive role in midbrain DA pathways. The organizational role of androgens in HPC was explored by Zhang and collaborators (2010) who reported that neonatal androgenic surges disruption increased depression-like behaviors in prepubertal male rats as well as reduced the number of MAP2 (microtubule-associated protein type 2)-immunopositive neurons in the dentate gyrus and the density of dendritic spines of the pyramidal neurons of the CA1 hippocampal areas.

Based on the existing literature and our own results, in the present study we antagonized the AR in the prenatal period in order to examine if the morphological consequences of gestational stress on the programing of the brain neural architecture of the offspring, might be related to the disruption of the perinatal androgen surge. We exposed pregnant rats to the non-steroidal drug flutamide during the last week of gestation which is a powerful and specific antiandrogen that crosses the placental barrier (Neri et al., 1972) and blocks AR by inhibiting its translocation to the nucleus from the cytoplasm of the target cells. We hypothesize that prenatal administration of flutamide might impair sexual maturation as well as brain morphology development in the prepubertal and adult offspring in a similar manner to the exposure of stress during late gestation that was previously reported by our group.

EXPERIMENTAL PROCEDURES

Animals

Eight virgin female Wistar rats weighing 250–280 g and sexually experienced Wistar male rats weighing 400–450 g were obtained from outbred rats belonging to the animal facility at the University of Buenos Aires. A maximum of four rats were housed per cage with *ad libitum* access to standard rat chow (*Asociación de Cooperativas Argentinas* – Buenos Aires, Argentina) and water. A constant light/dark cycle, with lights on at 06:00 h and off at 18:00 h, and a room temperature of 21–25 °C were maintained. Females were individually mated with a male in a mating cage. Vaginal smears were taken on the following morning. The day on which

spermatozoa were found in the smear was designated as day 1 of pregnancy. All procedures were in agreement with the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (Facultad de Medicina, Universidad de Buenos Aires). Care was taken to minimize the number of animals used.

Prenatal procedures and experimental design

Pregnant females were individually housed with *ad libitum* access to standard rat chow and water. A constant light/dark cycle (on at 06:00 h, off at 18:00 h) was maintained at a temperature of 21–25 °C. At GD 14, dams were randomly assigned to one of the following treatments:

- Vehicle (VEH): dams were transferred to an experimental room and injected s.c. with vehicle solution (5% ethanol–propylene glycol solution; 200 to 400 µl of solution according to body weight) from GD 15 to 21, between 9:00 and 10:00 h daily.
- Flutamide (FLU): dams were transferred to an experimental room and injected s.c. with a 10-mg/kg/day of flutamide solution (Flutamide obtained from Sigma Chemical Co. Ltd. St. Louis, dissolved in a 5% ethanol–propylene glycol solution) from GD 15 to 21, between 9:00 and 10:00 h (Dominguez-Salazar et al., 2002). We chose the dose of 10-mg/kg/day because the effects on male offspring sexual developmental parameters described in bibliography resemble those previously found on prenatally stressed descendants (Dominguez-Salazar et al., 2002; Goto et al., 2004; Pallares et al., 2013a).

Pregnant dam body weight belonging to both experimental groups was measured from GD 11 to 21 in order to explore the rate of daily body weight gain during pregnancy. An additional group comprised by undisturbed pregnant dams was also included in the analysis.

On the day of parturition, litter characteristics were recorded and culled to 10 pups. Four litters were maintained for each experimental group. Weaning was performed at postnatal day (PND) 21. The male and female offspring were housed in separate cages, with no more than five pups per cage, with standard rat chow and water *ad libitum*. Flutamide has been found to either have no effect in females or to further ''*feminize*'' females when given perinatally (Hammer, 1988; Brand and Slob, 1991; Grisham et al., 1992). Therefore only male offspring were used in this study. Throughout the paper, we will refer as *prepubertal* rats to PND 28 offspring, and *adult* rats to PND 75 offspring. To avoid litter effects, one pup from each litter was tested for each experiment. The timeline in Fig. 1 illustrates the experimental design.

Sexual developmental markers in male offspring

Sexual developmental markers were measured as described in Pallares et al. (2013a).

Anogenital distance. Anogenital distance was measured using a vernier-caliper on a randomly selected male pup from each litter at PND 1, 10 and 21 (Pallares et al., 2013a)

Testicular descent. Testicular descent was defined as the day when both testes were fully descended into the scrotal sac and could be palpated while the males were held vertically under their forelimbs. Rats were examined daily, starting on PND 21 (Pallares et al., 2013a). Results are expressed as the mean percentage of rats per litter that completed testicular descent, per day.

Body weight gain, organ-somatic index and testosterone concentration in serum. Body weight gain was measured at PND 1, 28 and 75. On PND 28 and 75, one male from each litter was decapitated within no more than 1 min after cage removal to avoid stress. Testes, epididymis and seminal vesicular glands were rapidly dissected and weighed. Organ-somatic index was calculated as: wet organ weight/corporal body weight × 100 (Olivares et al., 1996). Trunk blood was collected for testosterone levels in serum analysis. Blood was centrifuged and serum was used for measurement of testosterone concentration by radio immune assay. TESTO-RIA-CT Kit (DIA source Immuno Assays), showing 0.31% cross-reactivity with DHT, was used. The minimum detectable concentration for the assay was 0.05 ng/ml of testosterone and the intra-assay coefficient of variance was 4.6% (Pallares et al., 2013a)

Fixation and tissue processing

At PND 28 or PND 75 four males from different litters, belonging to VEH or FLU group, were deeply anesthetized with xylazine/ketamine hydrochloride solution (Mallinckrodt, 10-mg/kg and 75-mg/kg respectively). They were perfused through the cardiac left ventricle, initially with a cold saline solution containing 0.05% w/v $NaNO_2$ plus 50 IU of heparin and subsequently with a cold fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, post-fixed in the same cold fixative solution for 4 h, subsequently cryoprotected by overnight immersion in 15% and 30% w/v sucrose and stored at −80°C until their processing. Ten series of 25-µm-thick coronal sections for each area were cut on a Leica cryostat, according to anatomical landmarks corresponding to the Paxinos and Watson (1986) rat brain atlas. The sections were stored at −20 °C in 0.1 M phosphate buffer, pH 7.4, with 50% w/v glycerol added as a cryoprotector until their use in immunohistochemical or immunofluorescence studies.

The following areas were analyzed for immunohistochemistry: (1) layers II and III of PFC (plates 6–11, Paxinos and Watson (1986) rat brain atlas), (2) the *stratum radiatum* of the hippocampal CA1 area (plates 28–34), and (3) the VTA (plates 37–43). VTA was additionally analyzed for immunofluorescence.

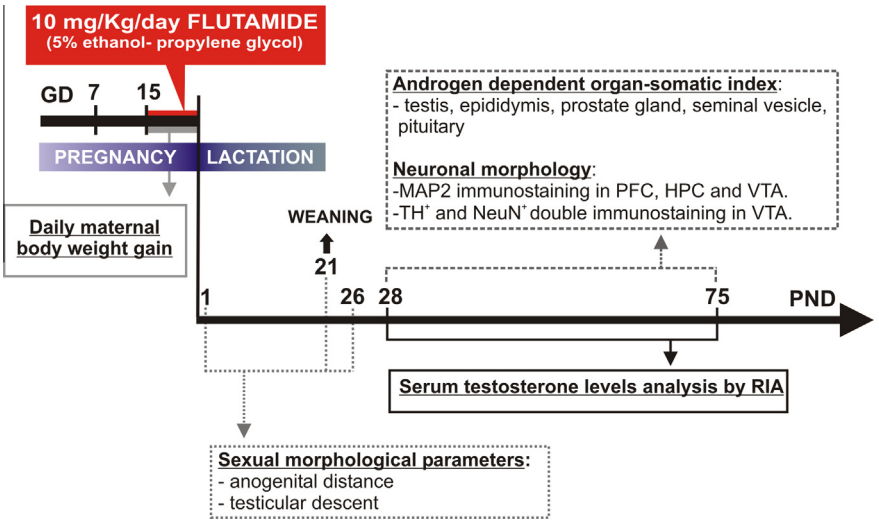

Fig. 1. Diagram of the experimental design. GD = gestational day, HPC = hippocampus, MAP2 = microtubule-associated protein type 2, NeuN = neuron-specific nuclear antigen, PFC = prefrontal cortex, PND = postnatal day, RIA = radio immune assay, TH = tyrosine hydroxylase, VTA = ventral tegmental area.

MAP2 immunohistochemistry

The sections were simultaneously processed in the free-floating state. To inhibit endogenous peroxidase activity, tissue sections were previously dehydrated, treated with 0.5% v/v H_2O_2 in methanol for 30 min at room temperature, and rehydrated. Brain sections were treated for 1 h with 3% v/v normal goat serum in phosphate-buffered saline (PBS) to block nonspecific binding sites. After two rinses in PBS plus 0.025% v/v Triton X-100 (PBS-X), sections were incubated for 48 h at 4 °C with primary antibodies to MAP2 (Synaptic System 188002; 1:1000). After further rinses in PBS-X, sections were incubated for 1 h at room temperature with biotinylated secondary antibodies diluted 1:200. After washing in PBS-X, sections were incubated for 1 h with streptavidin–peroxidase complex diluted 1:200. Sections were then washed five times in PBS and twice in 0.1 M acetate buffer, pH 6 (AcB), and development of peroxidase activity was carried out with 0.035% w/v 3,3′-diaminobenzidine hydrochloride (DAB) plus 2.5% w/v nickel ammonium sulfate and 0.1% v/v H_2O_2 dissolved in AcB. After the enzymatic reaction step, sections were washed three times in AcB and once in distilled water. Finally, sections were mounted on gelatine-coated slides; air dried, and cover slipped using Permount for light microscopic observation.

Image analysis of morphometric studies. To ensure objectivity, all measurements were performed on coded slides, under blind conditions, by two observers for each experiment, carrying out the measurements of MAP2-immunolabelled brain sections of both VEH and FLU rats with the same standardized observation schedule. Ten serial tissue sections were selected according to anatomical landmarks corresponding to the Paxinos and Watson (1986) rat brain atlas, for each area and for each animal. Total area of fibers was obtained with an Axiophot Zeiss light microscope equipped with a video camera online with a Zeiss-Kontron VIDAS image analyzer. The images were digitized and processed in a resolution of 256 gray levels for each pixel. The morphometric studies using immunolabelled structures for cytoskeletal proteins were performed as previously described (Pallares et al., 2013b; Ramos et al., 2000). To evaluate the MAP2 immunoreactivity fibers, the total area of the immune labeled fibers was related to the total area of the corresponding microscopic field within a defined sampling box which consisted on a 400×300 μm area for PFC or 200×200 μm area for HPC and VTA, thus rendering a relative area of fiber density parameter. Image ProPlus software was employed to select pixels within the sampling box that matched threshold criteria for MAP2-positive immunostaining. The threshold was set at the level that selected the lightest stained cell bodies and dendrites without selecting background staining. The setting for illumination was kept constant throughout the analysis. The computer outlined and added the threshold areas and determined the ratio of the stained area to the total area of the sampling box. If there were histological artifacts in the tissue, then the box was placed at the next straight edge after the artifact to avoid distortion (Marmolejo et al., 2012).

Immunofluorescence staining

In order to identify DA neurons double immunofluorescence staining for neuron-specific nuclear antigen (NeuN) and tyrosine hydroxylase (TH) was carried out on free-floating VTA sections. Briefly, PBS was used to rinse sections before they were incubated for 1 h in 3% v/v normal goat serum in PBS to block non-specific binding sites at room temperature under constant agitation. Sections were incubated overnight under agitation with anti-NeuN (1/1000, clone AG0, MAB377, Millipore, USA) and anti-TH (1/1000, P40101-0, Pel-Freez, Rogers, AR, USA). Following rinses in 0.025% PBS-X, the slices were

incubated with the following antibodies: mouse anti-IgG conjugated with Alexa fluor 488 (1/1000, A-11001, Invitrogen, Buenos Aires, Argentina) and rabbit anti-IgG conjugated with cy3 (1/250, 711-165-152, Jackson Immuno Research Laboratories, Inc. West Grove, PA, USA). The antibodies were diluted in 0.3% Triton X-100 and 1% normal goat serum in PBS. Sections were mounted on gelatine-coated microscope slides and covered-slipped using immunofluorescence media (S3023, DAKO, USA) for microscope observations.

Stereological counts of NeuN+ and TH+ cells. All processed sections were examined and digitally photographed on an Olympus BX50 microscope equipped with a digital camera Cool-Snap and the image analyses were performed by the use of the Image ProPlus program. For each rat, the number of NeuN+ and TH+ cells was determined in both brain hemispheres from six coronal sections encompassing rostral, middle, and caudal levels of the VTA. The boundaries of the structures in the coronal plane were determined microscopically (40× total magnification). Since measurements were made on every one of eight (for PND 28 rats) or one of 10 (for PND 75 rats) serial sections (i.e., separated by 600 μm), it was unlikely that the same neuron would be analyzed twice. NeuN+ and TH+ cells were counted on a single immunofluorescence picture and subsequently the co-localizing cells were counted on merging images from two separate channels without altering the position of the sections or focus, by using the Image ProPlus software.

NeuN+ cells and the merge of the photomicrographs obtained in a same field for both markers (i.e. TH+ NeuN+ cells) were counted at a 200X total magnification from a rectangular area of 400 × 300 μm per hemisphere, per section. The number of DA neurons was expressed as the% of TH+ NeuN+ cells/ total NeuN+ cells × 100.

Statistical analysis

Litter characteristics analyses, organ-somatic index, immunohistochemistry and immunofluorescence results were analyzed by *t*-Student test. Anogenital distance and testosterone results were analyzed by two-way analysis of variance (ANOVA) in order to evaluate the effects due to prenatal treatment, age and possible interactions between both factors. When interactions were found, simple effects ANOVA analyses were done. The statistical analyses of the daily maternal body weight gain during pregnancy and testicular descent data were performed by repeated measures ANOVA. Visual inspection of histograms, qq plots and random distribution of fitted values were checked. All results are presented as mean ± SEM. The observed differences were considered to be statistically significant when $p < 0.05$. *n* values reported in figures represent number of litters. Analysis of data was performed by using SPSS 13.0 version and Infostat 2013.

RESULTS

Effect of flutamide administration on pregnant dams body weight gain

Maternal daily body weight gain was evaluated from GD 12 to 21 in VEH- and FLU-injected dams. An additional control group of undisturbed pregnant dams was also included in the analysis (Fig. 2). In the three experimental groups the daily body weight gain of pregnant dams increased according to the gestation progression (*repeated-measures ANOVA*: $F_{(6,6)} = 14.89$, $p < 0.001$ for gestational day factor). No statistical differences between experimental groups were observed in the rate of daily body weight gain (*repeated-measures ANOVA*: $F_{(1,5)} = 0.58$, $p = 0.4806$ for treatment factor).

Litter parameters and male pup physical characteristics

The analysis of litter parameters on the day of birth revealed that FLU did not interfere with the length of gestation, number of pups or body weight gain from birth to adulthood (Table 1). Nor missing limbs or gross malformations were found in any of the new born pups. However, the external appearance of the genitalia and the scrotal area were indistinguishable between females and males offspring at the moment of birth. Furthermore,

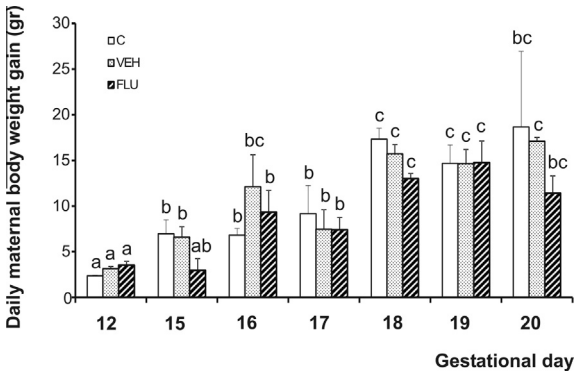

Fig. 2. Daily maternal body weight gain during late pregnancy in control undisturbed, vehicle and flutamide-injected females. C = control, FLU = flutamide, VEH = vehicle. Values represent the mean of the daily body weight gain ± SEM. Groups with no letters in common are significantly different with $p < 0.05$ ($n = 4$; repeated measures ANOVA-Tukey).

Table 1. Litter characteristics. Four different litters were evaluated for each parameter (*t*-student test)

Evaluated parameter	Vehicle	Flutamide
Length of gestation (days)	22.75 ± 1.08	23 ± 0.0
Litter size	12.75 ± 2.99	14 ± 2.45
Pups body weight at birth (gr)	6.67 ± 1.03	7 ± 1.1
Pups body weight at PND 28 (gr)	85 ± 12.02	79.99 ± 4.00
Pups body weight at PND 75 (gr)	326.7 ± 6.00	348.8 ± 7.6

all prenatal flutamide-exposed rats had mammillary buds. In many cases, the sex of the flutamide-exposed pups could not be determined until PND 4–6, when differences in the genital bud appearance between sexes could be determined. Females offspring sexual characteristics were unaffected by treatment (data not shown). However changes in sexual development of males offspring exposed *in utero* to flutamide were displayed. A significant reduction in the anogenital distance for FLU males was observed at all evaluated ages in comparison to VEH males (Fig. 3A, B; *two-way ANOVA*: $F_{(1,57)} = 168.2$, $p < 0.001$ for prenatal treatment factor effect). In addition, the anogenital distance found in FLU males was comparable to that found on FLU females (*two-way ANOVA*: $F_{(1,57)} = 9.4 \times 10^{-5}$, $p = 0.9923$ for sex factor effect).

Penis hypospadias was observed in all FLU males: the urethral orifice was placed basally and displayed a higher diameter than VEH male penis (Fig. 3A arrows in PND 21 VEH male vs. PND 21 FLU male). Vaginal pouch was also observed at PND 21 FLU males. Moreover, while most of the VEH rats completed testis descent at PND 23, FLU rats showed a delay by two days (Fig. 3C, D; *repeated-measures ANOVA*: $F_{(5,30)} = 18.55$, $p < 0.001$ for age factor effect, and $F_{(1,5)} = 12.09$, $p = 0.0132$ for prenatal treatment effect). Moreover a minor proportion of FLU rats presented cryptorchidism both at PND 28 and PND 75 (arrows in Fig. 3C).

Testes, epididymis and seminal vesicle organ-somatic index were evaluated in prepubertal and adult VEH and FLU rats. No changes on the organ-somatic index were found on testes or epididymis, but uncompleted development of the seminal vesicles and absence of the coagulating gland were observed in almost all FLU rats, while no such abnormalities were identified in VEH ones (Fig. 4A; *t*-student test: $T_{(6)} = -10.13$, $p = 0.0005$ VEH vs. FLU for seminal vesicles somatic index at PND 28; $T_{(6)} = 5.52$, $p = 0.0015$ VEH vs. FLU for seminal vesicles somatic index at PND 75).

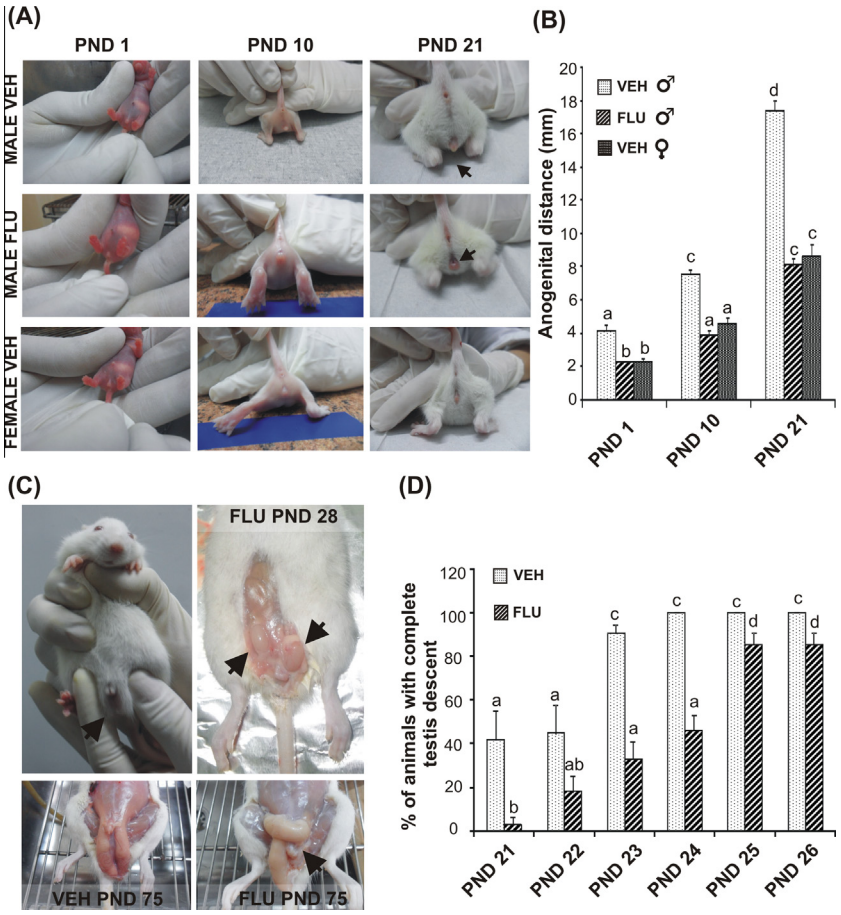

Fig. 3. Effects of prenatal flutamide administration on the anogenital distance and testicular descent. FLU = flutamide, PND = postnatal day, VEH = vehicle. (A) Photographs show representative anogenital distances observed in VEH or FLU males and VEH females at PND 1, 10 and 21. Arrows depict penis morphological differences between VEH and FLU males at PND 21. (B) Values are reported as the mean anogenital distance ± SEM. Groups with no letters in common are significantly different with $p < 0.05$ ($n = 6$; two-way ANOVA-Tukey). (C) Upper photographs correspond to a PND 28 rat with an undescended right testis into the scrotal sac. Lower photograph on the left side corresponds to a PND 75 VEH rat. Lower photograph in the right side corresponds to a PND 75 FLU rat with cryptorchidism in the left testis (depicted by the arrow). (D) Values represent the mean percentage ± SEM of rats per litter in which both testes descended into the scrotal sac. Groups with no letters in common are significantly different with $p < 0.05$ ($n = 4$ litters analyzed per group; repeated measures ANOVA-Tukey).

Serum testosterone concentrations assay

No significant differences between VEH and FLU groups were observed on specific serum testosterone concentrations in prepubertal or adult rats (Fig. 4B).

MAP2 immunoreactivity

MAP2 immuno-density of each analyzed area was calculated by carefully matching VEH and FLU sections in the same antero-posterior plane to avoid differences in cell distribution along the rostro-caudal axis. The immuno-labeled processes were observed as round stains or longitudinal tracts, according to whether dendrites were transversally or tangentially sectioned. Both in prepubertal and adult VEH rats relative area of the MAP2-immunostained processes was observed to be abundant in all areas studied, but was scarce in FLU rats at both evaluated ages (Figs. 5–7). Semi quantitative analysis of these results showed that the decrease was statistically significant. In PFC, a decrease of 16% and 15% of MAP2-immunostaining at PND 28 and at PND 75 FLU rats respectively was found (Fig. 5; *t*-student test: $T_{(6)} = -2.83$, $p = 0.0298$ VEH vs. FLU at PND28 and $T_{(6)} = -5.34$, $p = 0.0128$ VEH vs. FLU at PND 75).

MAP2-immunostaining was evaluated in the CA1 region of the HPC. Prenatal administration of flutamide decreased MAP2 48% in prepubertal rats and 20% in adult rats, in comparison of the MAP2-immunostaining registered in VEH rats (Fig. 6; *t*-student test: $T_{(6)} = -6.06$, $p = 0.009$ VEH vs. FLU at PND28 and $T_{(6)} = -4.02$, $p = 0.0101$ VEH vs. FLU at PND 75).

Finally, MAP2-immunolabel in the VTA of FLU offspring at PND 28 presented a reduction of 24%, while adult FLU rats presented a reduction of 31% in MAP2, comparing to MAP2-immunolabel quantified in their VEH counterparts (Fig. 7; *t*-student test: $T_{(6)} = -4.89$, $p = 0.0045$ VEH vs. FLU at PND28 and $T_{(6)} = 3.74$, $p = 0.0097$ VEH vs. FLU at PND 75).

Co-localization of NeuN and TH in offspring VTA

Total number of DA neurons in VEH and FLU offspring was quantified in VTA, by using the antibody anti-NeuN which is a specific neuronal cell marker and the antibody anti-TH as a specific DA marker (Engele et al., 1989). TH is a key enzyme in the synthesis of dopamine. Therefore, co-localization of both markers reveals DA neurons.

NeuN-immuno label was observed as a homogeneous nuclear stain in all cells in the section, while TH-immuno reactivity was seen in the cytoplasm and in the neural processes of the cells belonging to substantia nigra and VTA areas. No statistical differences were observed among groups in the number of NeuN+ cells at PND 28 or PND 75 rats (Figs. 8 and 9A). Quantification of TH+ cells co-expressing NeuN in the VTA revealed that the percentage of TH+/NeuN+ was 35% lower in PND 28 FLU rats in comparison of their VEH counterparts (Figs. 8 and 9B; $T_{(6)} = -2.71$, $p = 0.0329$). There were no significant differences between groups at PND 75.

DISCUSSION

Previous studies from our group have suggested that impairments on the organizational effects of androgens during late gestation might be implicated in the long-term consequences of prenatal stress on the

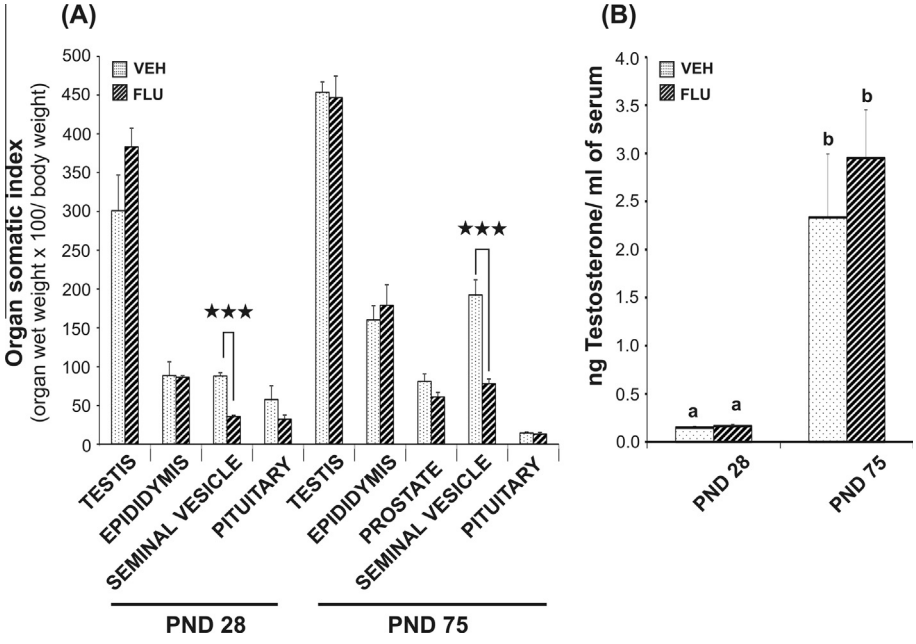

Fig. 4. Effects of prenatal flutamide administration on androgen-dependent organ-somatic index and on serum testosterone concentrations. FLU = flutamide, PND = postnatal day, VEH = vehicle. (A) Values represent the mean ± SEM of testis, epididymis, seminal vesicle and pituitary somatic index (which was calculated as: wet organ weight/body weight × 100). Stars show the presence of statistical differences among groups ($n = 4$; $^{***}p < 0.001$; *t*-student test). (B) Values represent the mean ± SEM of serum testosterone concentrations ($n = 4$; two-way ANOVA).

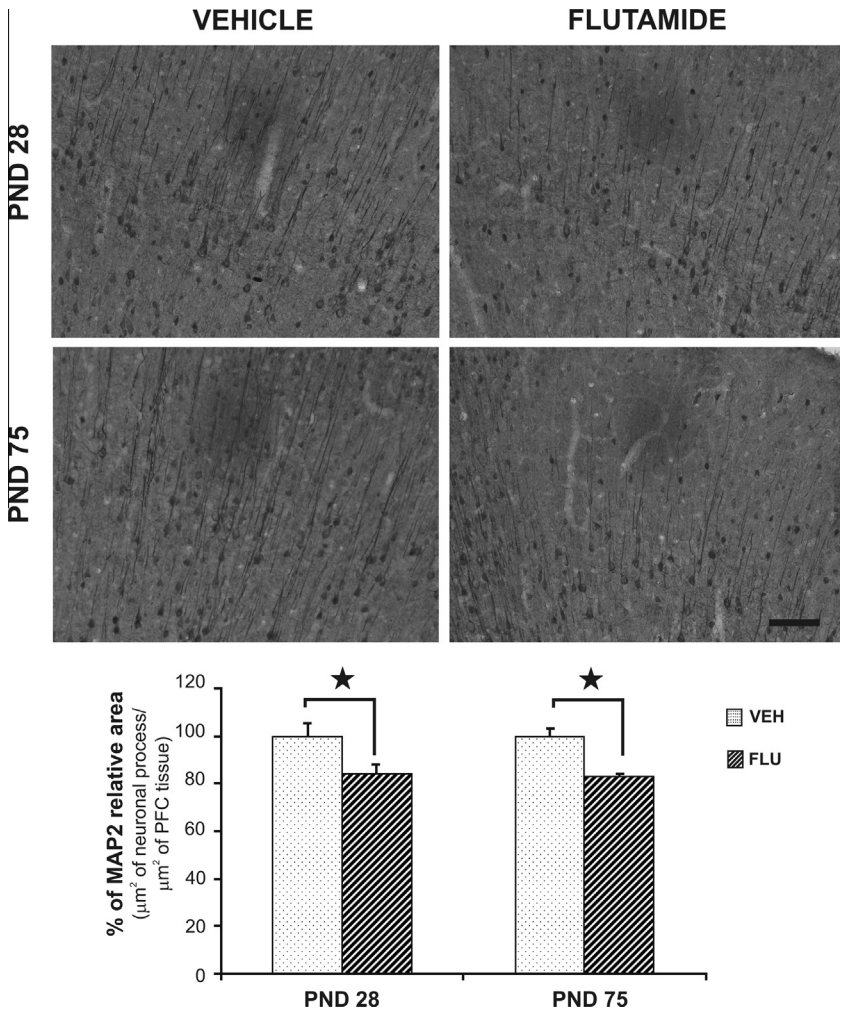

Fig. 5. Effects of prenatal flutamide on MAP2 immunoreactivity at layers II and III of the prefrontal cortex (PFC). FLU = flutamide, PND = postnatal day, VEH = vehicle. Micrographs show examples of immunostaining with an anti-MAP2 antibody in PFC for each experimental group (Plate 10 according Watson and Paxinos (1986) Rat Brain Atlas; 200× total magnification; scale bar = 100 μm). Values are reported as mean ± SEM of the MAP2 immunoreactivity in PFC (μm^2 neuronal processes/μm^2 tissue). Star demonstrate the presence of statistical differences between experimental groups ($n = 4$; $^*p < 0.05$; *t*-student test).

morphology of certain brain areas that are involved in the control of major behaviors such as the PFC, HPC and VTA in the male rat offspring (Baier et al., 2012; Pallares et al., 2013a,b). In the present work, we blocked AR during *the critical period* of brain differentiation by administering flutamide to the pregnant dam on the assumption that flutamide changes might resemble prenatal stress consequences on the organization of the PFC, HPC and VTA neuroarchitecture in male rats. Treatment with flutamide allows for a reversible androgen blockade. However testosterone may continue to act at receptors through its aromatization to estradiol. Main findings of this study highlight that androgens acting during late gestation not only modulate the sexual maturation of the progeny external genitalia but they are also involved in the development of the offspring brain by inducing long-term changes on the neural morphology that mimics the effects observed previously in our group by prenatal stress.

Effects of prenatal flutamide administration on daily maternal body weight gain during pregnancy and on offspring sexual development

Animal studies indicate that the exposure of adult subjects to chronic stressful events induces a long-term reduction in the body weight gain due to the catabolic action of glucocorticoid hormones over muscle and adipose tissues (Smagin et al., 1999). Unpublished data from our group demonstrated that restraint stress exerted on the pregnant rat from GD 14–21 reduced daily body weight gain and overall body weight growth of the pregnant dam during the stressful protocol. This is in agreement with Darnaudery and collaborators (2004), who further showed this reduction even until weaning. In this work, body weight gain was similar in undisturbed, VEH- and FLU-injected dams, implying that the injections might not be perceived as a stressful situation by the pregnant rat. The absence of differences in the daily body

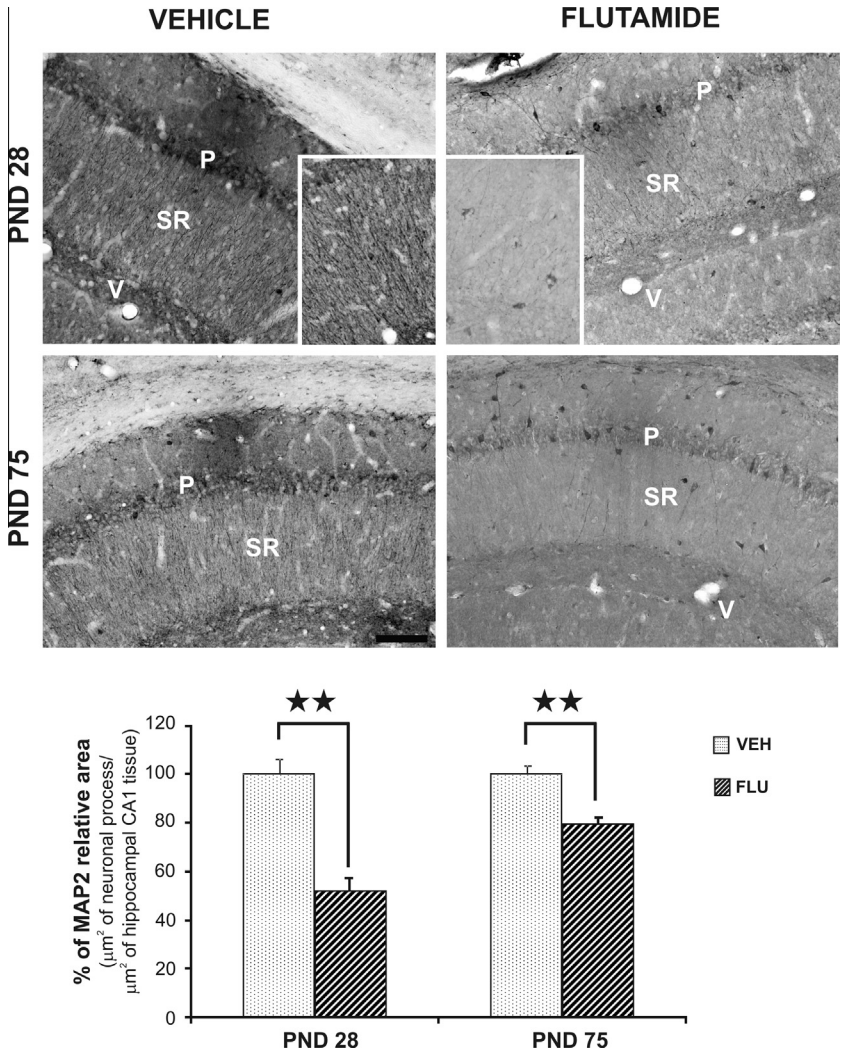

Fig. 6. Effects of prenatal flutamide on MAP2 immunoreactivity at CA1 hippocampal area. FLU = flutamide, P = pyramidal neuronal layer, PND = postnatal day, SR = stratum radiatum, V = vein vessel, VEH = vehicle. Micrographs show examples of immunostaining with an anti-MAP2 antibody in CA1 hippocampal area for each experimental group at PND 28 and at PND 75 (Plate 31 according Watson and Paxinos (1986) Rat Brain Atlas 200× total magnification; 400× total magnification in insets detailing stratum radiatum area; scale bar = 100 μm). Values are reported as mean ± SEM of the MAP2 immunoreactivity in CA1 (μm^2 neuronal processes/μm^2 tissue). Stars demonstrate the presence of statistical differences between experimental groups ($n = 4$; $^{**}p < 0.01$; *t*-student test).

weight gain observed among groups might reinforce the fact that the observed effects on the progeny were due to the anti-androgenic effect of flutamide and not to a stressful event perception of the dam during last week of pregnancy. Nevertheless an extended evaluation of the pre and post-gestational maternal status body weight gain should be considered in future studies.

The examination of morphometric parameters, in particular the anogenital distance and testicular descent into the scrotum, provides simplest and least invasive indicators of normal androgen exposure during the masculinization programing window (Scott et al., 2009). Anogenital distance is generally twofold greater in males than in females, and the testicular descent normally occurs at PND 21 in rats. In most mammals, the testes must descend from the abdomen to an extracorporeal position to provide a lower ambient temperature for normal spermatogenesis. In the rat, the release and movement of the testes into the scrotum occur in two phases: the *trans-abdominal phase* that takes place during fetal life and is controlled by the insulin-like 3 factor which is produced by the fetal Leydig cells under the control of LH. The second phase, termed *inguino-scrotal phase*, is controlled by androgens and takes place after birth with the neonatal surge in testosterone levels (Goto et al., 2004; Hughes and Acerini, 2008). Insufficient testosterone production or action during the masculinization programing window in the fetal rat can result in disorders of masculinization including malformation of the penis, cryptorchidism and reduced anogenital distance. In the present study, and in concordance with Casto and collaborators (2003), the administration of flutamide during the last week of gestation reduces the anogenital distance in males at all evaluated ages. Moreover, from birth to PND 40 (data not shown) there were no differences in the anogenital distance between FLU males

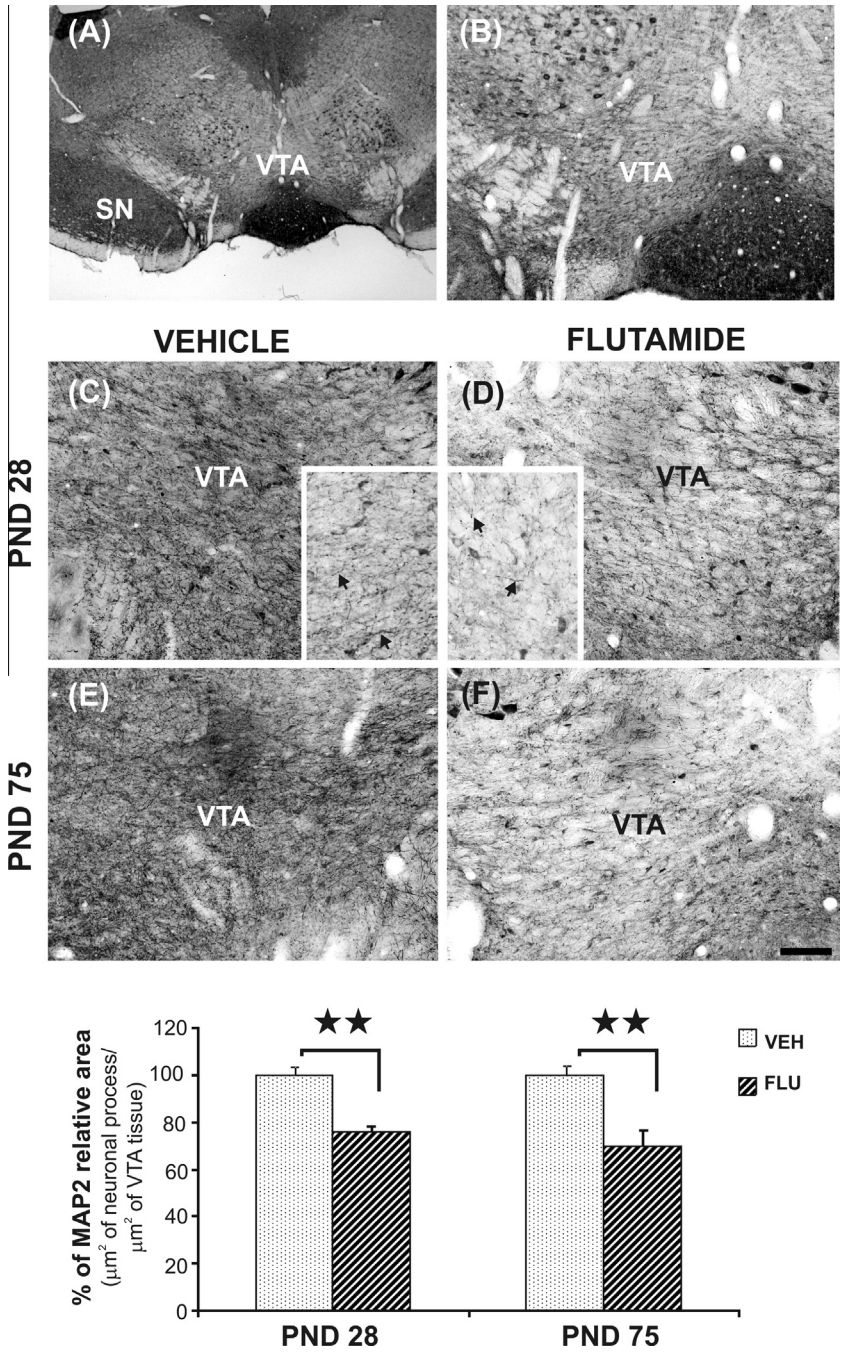

Fig. 7. Effects of prenatal flutamide on MAP2 immunoreactivity at ventral tegmental area (VTA). FLU = flutamide, PND = postnatal day, SN = sustantia nigra, VEH = vehicle. (A, B) Two panoramic micrographs of the studied area (Plate 40 according Watson and Paxinos (1986) Rat Brain Atlas; A: 40× and B: 10× total magnification). (C–F) Micrographs show examples of immunostaining with an anti-MAP2 antibody in VTA for each experimental group (200× total magnification; 400× total magnifications in insets; scale bar = 100 μm). Arrows in insets show MAP2-immunopositive mark as longitudinal tracts. Values are reported as mean ± SEM of the MAP2 immunoreactivity in VTA (μm^2 neuronal processes/μm^2 tissue). Stars demonstrate the presence of statistical differences between experimental groups (n = 4; $^{**}p < 0.01$; t-student test).

and females offspring. Malformation of the penis was also observed in FLU rats. This is in agreement with Imperato-McGinley and collaborators (1992) who reported the presence of non-functional blind vaginal pouches (Casto et al., 2003; Goto et al., 2004). As previously reported by Goto and collaborators (2004) and Okur et al. (2006), we also observed that prenatal flutamide induces a delay in the testicular descent of FLU males, while a small percentage of rats per litter presented cryptorchidism (i.e. they maintained one of the testes on the abdominal cavity even at PND 75). The intra-litter variations in the incidence of cryptorchidism found in our work may indicate that fetuses become exposed to ''maternal'' flutamide with variable efficiency. There may be subtleties in the maternal or placental circulation causing differences in the degree of exposure to the drug (van der Schoot, 1992). It has

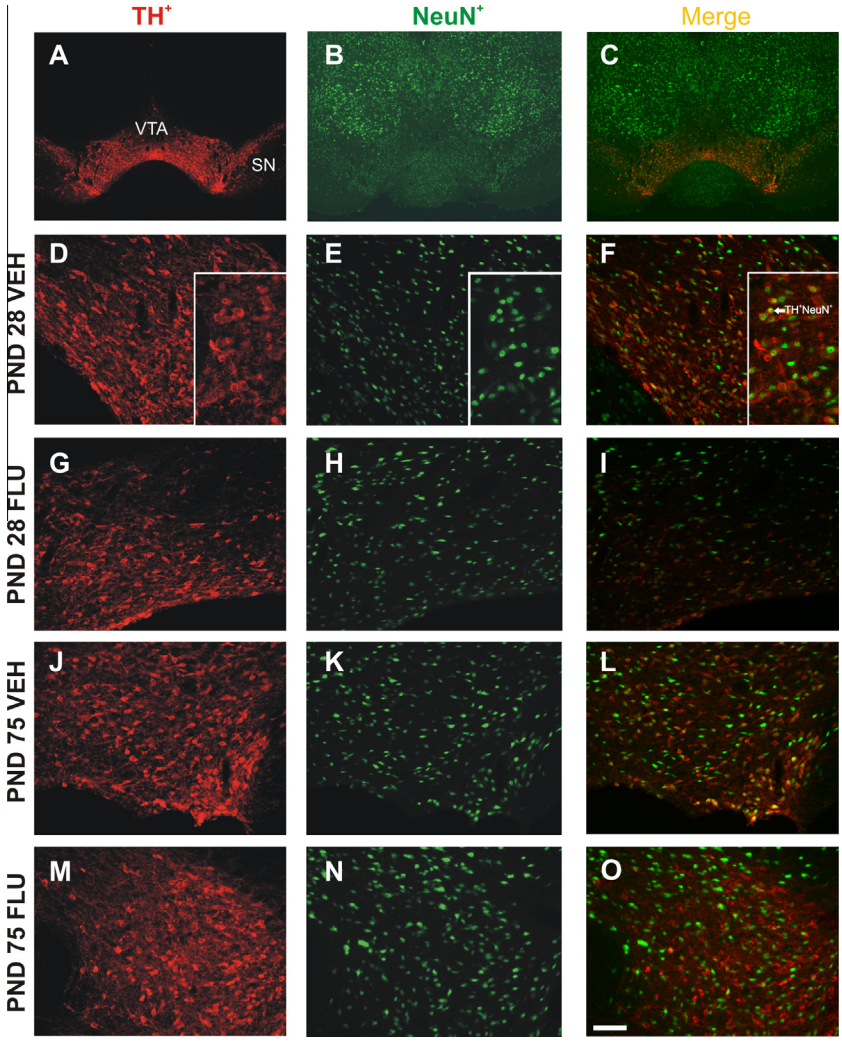

Fig. 8. Effects of prenatal flutamide on double immunofluorescence of neuron-specific nuclear antigen (NeuN) and tyrosine hydroxylase (TH) at the ventral tegmental area (VTA). FLU = flutamide, PND = postnatal day, SN = substantia nigra, VEH = vehicle. (A–C) Micrographs show panoramic examples of NeuN, TH and the merge of both markers (40× total magnification). (D–O) Micrographs show representative immunostaining with an anti-NeuN and anti-TH antibodies in VTA for each experimental group (200× total magnification). Details could be observed at a 400× total magnifications in the insets in (A–C) microphotographs. White arrow in (C) depicts an example cell with an immunopositive label for both markers. Scale bar = 100 μm.

been described that prenatal administration of flutamide induced a reduction on the testicular weight, as well as on epididymis, prostate gland and seminal vesicles. In this work we only registered differences in the organ-somatic index of seminal vesicles of both prepubertal and adult FLU rats. Atrophied seminal vesicles were found at both ages. The absence of the effect of fetal flutamide exposure on testis and epididymis weight could be explained by the inability of flutamide to effectively antagonize androgen action within the fetal testis because of the high levels of intra-testicular testosterone concentrations (Scott et al., 2009).

The concentrations of testosterone in the serum did not differ between VEH or FLU offspring at both evaluated ages. Thus, prenatal antiandrogen treatment during the last week of gestation seems not to interfere with the mechanisms involved in the pubertal hormonal surges. In concordance with our results, other authors found no differences between experimental groups in testosterone serum concentrations in adult offspring (Casto et al., 2003; Goto et al., 2004).

Effects of prenatal flutamide administration on PFC, HPC and VTA neural morphology

Since normal brain function development depends on the maturation of synaptic circuits that takes place in dendritic spines, (Segal, 2010), we explored dendritic density by assessing MAP2 immunostaining. MAP2 is the major MAP in the brain, and is highly localized to dendrites, where it can be visualized and quantified immunehistochemically. Its expression coincides with neurite outgrowth and dendritic branching (Chamak et al., 1987). In this study, we demonstrate that the administration of flutamide during the gestational period induced a long-term reduction in MAP2-immunostaining in offspring PFC, CA1 hippocampal region and VTA suggesting that flutamide reduced dendritic arborizations of these areas.

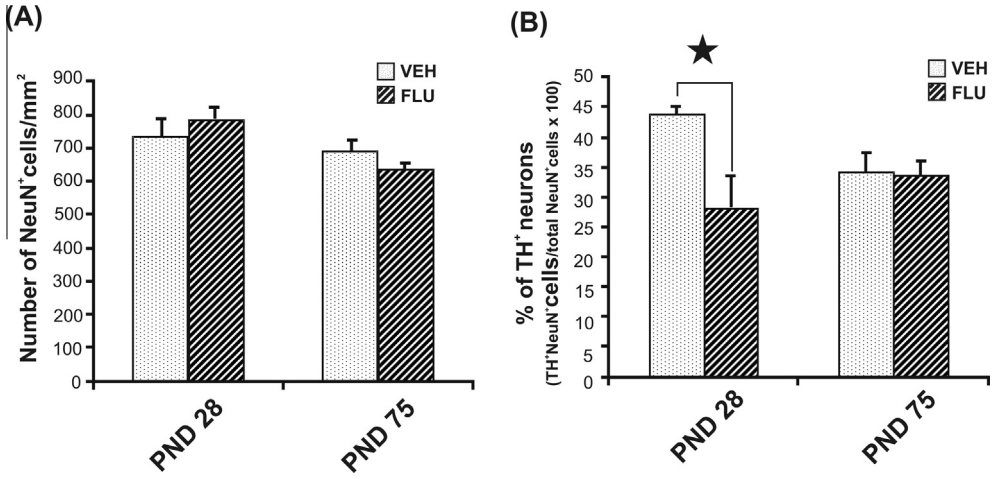

Fig. 9. Quantification of neuron-specific nuclear antigen (NeuN) and tyrosine hydroxylase (TH) co-expressing cells at the ventral tegmental area (VTA). FLU = flutamide, PND = postnatal day, VEH = vehicle. Values are reported as mean ± SEM of the number of NeuN-immunopositive cells (A) or dopaminergic neurons (B). The number of dopaminergic neurons was evaluated by examining the percentage of cells expressing both markers/total number of neurons * 100. Star demonstrate the presence of statistical differences between experimental groups ($n = 4$; $^*p < 0.05$; *t*-student test).

Previous studies from Zhang and collaborators (Zhang et al., 2010) demonstrated that neonatal flutamide administration induced plastic changes in CA1 and dentate gyrus hippocampal regions of prepubertal offspring. Moreover, these findings on brain morphology were related to increased depressive-like behaviors in FLU rats.

It is interesting to point out the similarities of these results with the prenatal stress paradigm. Previous studies from our group showed that prenatal stress induced a reduction of the MAP2-immunoreactivity in PFC and HPC of prepubertal and adult offspring (Barros et al., 2006; Pallares et al., 2013b) suggesting that prenatal stress as much as prenatal flutamide administration, might be disrupting the normal neuronal processes development which normally takes place from puberty to adult life (Huttenlocher, 1979). It was postulated that the role of neonatal androgens on the developing neuronal processes is to prepare neurons to react upon specific environmental cues which are in turn responsible to redirect the development of dendritic arborizations later in life. Neonatal flutamide administration inhibits such androgen organizational effects. Moreover, the testosterone surge that physiologically occurs during puberty might be insufficient to reverse this effect (Zhang et al., 2010).

Effects of prenatal flutamide administration on TH+ neurons in VTA

Numerous studies demonstrated that androgens affect TH at multiple levels. It was reported that *in vivo* treatment with testosterone increased the expression of TH in different brain areas (Weltzien et al., 2005) and that AR could positively regulate the transcription of the TH gene suggesting the existence of a positive interrelation between dopamine and androgens (Jeong et al., 2006). On the other hand, androgens appeared to exert suppressive effects on the enzyme expression in mesencephalic structures since increased TH expression were found on substantia nigra and VTA of adult male rats which were gonadectomized at adulthood. Such increase was inhibited if gonadectomized animals were additionally supplied with testosterone or DHT (Johnson et al., 2010). Even though the activational role of androgens on TH was explored in the literature, little research was done on the organizational role of hormones on TH. It was observed that perinatal subtraction of gonads induced a reduction in the TH-immunolabel at the anterior cingulated cortex of adult rats, but such effects were attenuated if gonadectomized rats were supplemented with testosterone (Kritzer, 1997).

In our hands, the offspring that were prenatally exposed to flutamide showed no differences in the total number of neurons in VTA but presented a reduction on the percentage of immunopositive TH neurons at PND 28 but not at PND 75. The effective reduction in TH+ cell number by flutamide in prepubertal rats suggests that testosterone and/or DHT might be mediating these effects. Since, in our study, prepubertal and adult testosterone levels were not affected by prenatal flutamide, the restitution of TH expression at PND 75 FLU rats might be related to the testosterone surge that normally occurs during puberty. However, to confirm this hypothesis, flutamide should be administered prepubertally in order to suppress the testosterone pubertal surge and therefore confirm the TH-immunopositive cells restitution in VTA of adult offspring by the hormonal action. Future studies might also contemplate a higher number of subjects to measure testosterone concentrations as well as exploring other hormonal systems that might be interfering. These experiments were out of the scope of the present study but they will undoubtedly help to clarify the mechanisms involved in the organizational/activational sexual hormones effects on brain morphology.

CONCLUSIONS

The last week of gestation in rats constitute a critical period of masculinization of several androgen-dependent anatomical features that include the external

genitalia and the sexual differentiation of the brain. During this critical time androgens mediate the establishment of a proper neural substrate that determines brain function during adult life. Main findings of the current study show that prenatal blockade of ARs induces long-term alterations of the correct organization of morphological and neurochemical features of brain development. Prenatal flutamide induced a long-term decrease in MAP2-immunoreactivity in PFC, HPC and VTA, as well as reduced the number of immunopositive TH neurons in VTA only at prepubertal rats, suggesting that some plastic processes are programed prenatally and androgens play an important role on such brain orchestration. The results obtained in this work resemble previous results obtained by our group on prenatally stressed offspring suggesting that several effects of prenatal stress on developing brain might be explained by the disruption of the organizational effects of androgens during critical phases of brain development.

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