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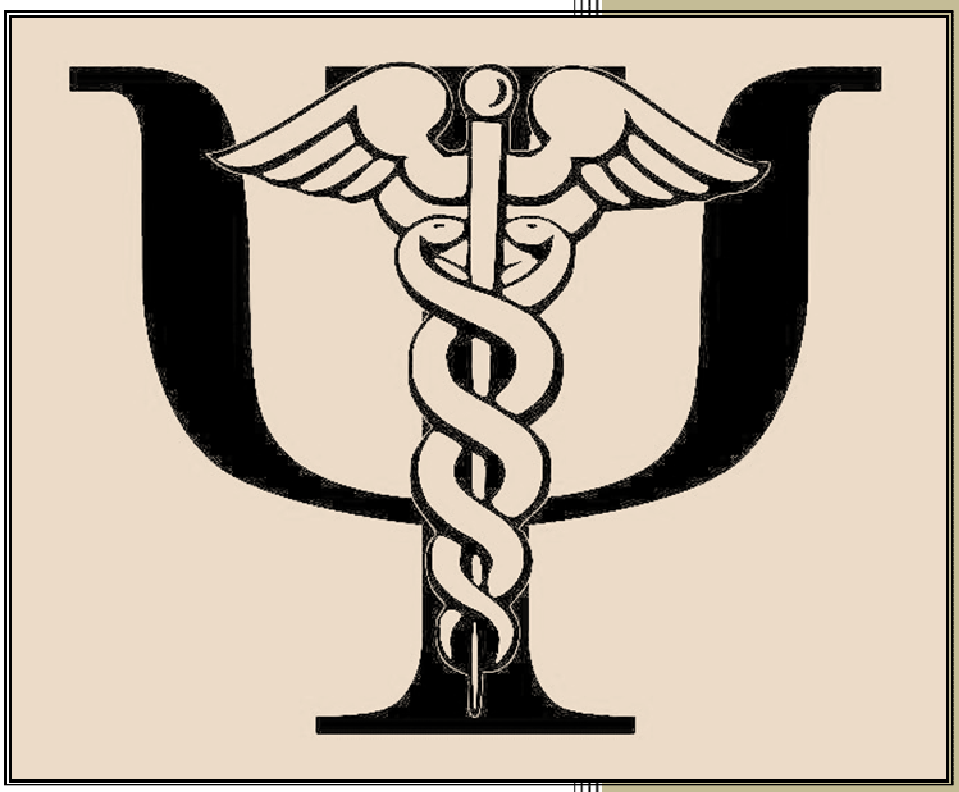

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EFFECTS OF AROMATASE INHIBITION ON SPATIAL WORKING MEMORY AND HIPPOCAMPAL ASTROCYTE NUMBERS

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Abstract

Sex hormones are known to induce the sexual differentiation of the brain during early development in mammals. Testosterone secreted by males already during gestation is classically believed to contribute to brain and behavioural sexual differentiation thanks to its conversion to estradiol by the enzyme aromatase. However, there is evidence suggesting that aromatase inhibition may also impair cognitive functions in women receiving hormonal treatment for breast cancer. In order to evaluate the effects of aromatase on brain and behaviour, male and female prepubescent rats treated with anastrozole prenatally and during early postnatal development were tested in a spatial working memory task. Results show that anastrozole treatment clearly impaired spatial working memory in male and female rats as compared to sex-matched vehicle-treated and control groups. In addition, the number of astrocytes expressing glial fibrillary acidic protein (GFAP) decreased in the CA3 area of the dorsal hippocampus only in male rats. These results indicate that aromatase plays a complex role on the sexual differentiation of the brain and affects spatial memory in males and females.

Keyworks: GFAP-ir; aromatase; anastrozole; hippocampus; stereology; rat.

Resumen

Se sabe que las hormonas sexuales inducen la diferenciación sexual del cerebro durante el desarrollo temprano en mamíferos. La testosterona secretada por los machos ya durante la gestación está tradicionalmente asociada con la diferenciación sexual cerebral y conductual, gracias a su conversión a estradiol por el enzima aromatasa. Sin embargo, hay evidencia de que la inhibición de la aromatasa puede también deteriorar las funciones cognitivas en mujeres que reciben tratamiento hormonal para el cáncer de mama. Con el fin de estudiar los efectos de la aromatasa en el cerebro y la conducta, ratas prepúberes macho y hembra tratadas prenatalmente con anastrozol y durante el desarrollo temprano fueron evaluadas en una prueba de memoria de trabajo espacial. Los resultados muestran que el tratamiento con anastrozol deterioró claramente la memoria de trabajo en machos y hembras en comparación con grupos tratados con vehículo y control. Además, el número de astrocitos que expresaron la proteína glial fibrilar ácida (GFAP) disminuyó en el área CA3 del hipocampo dorsal sólo en ratas macho. Estos resultados indican que la aromatasa juega un papel complejo en la diferenciación sexual del cerebro y afecta a la memoria espacial en machos y hembras.

Palabras clave: GFAP-ir; aromatasa; anastrozol; hipocampo, estereología; rata.

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Introduction

It is widely accepted that the sexual differentiation of the brain is solely dependent on androgens synthesized in the fetal and neonatal testicles and converted to estradiol by the enzyme aromatase in the nervous system during the perinatal period. Testosterone and its metabolite dihydrotestosterone are the main gonadal or sex steroid hormones in males. In addition, aromatase is a key enzyme in estrogen biosynthesis that catalyzes the conversion of testosterone to estradiol and locally synthesized steroids known as neurosteroids (Stoffel-Wagner, 2001). Neurosteroids have numerous functions in the brain not fully understood, although they can modify neuronal metabolism and affect behavior. Conversely, testosterone plasma levels are low in the perinatal female rat (Hansen, Södersten, Eneroth, Srebro, & Hole, 1979). However, estradiol levels in male brain are high because gonadal testosterone is converted to estradiol by aromatase (Konkle & McCarthy, 2011). Therefore, aromatase is a key enzyme involved in the organizational and activational effects of sex steroids on the developing brain. Moreover, aromatase expression has sex-related differences in particular brain regions during the perinatal period of rats (Colciago et al., 2005).

Anastrozole is a non-steroidal drug acting as an aromatase inhibitor widely used to treat hormone-dependent breast cancer in postmenopausal women. Anastrozole decreases in situ conversion of androgens to estrogens. However, few and conflicting studies have directly addressed the effects of aromatase inhibition on brain and behavior. One of the most studied brain regions as regards to the effects of sex steroids on behavior is the hippocampus. The hippocampus is critically involved in a range of behaviors, including learning, memory, stress, fear, and depressive and anxiety disorders, all of which vary to some degree between males and females (Bale, 2006; Goldstein, 2006). There is mounting evidence that sex steroid hormones, including estrogens and androgens, play important roles in hippocampal dimorphism both anatomically and functionally (McEwen, 1983; McEwen, 1999, Pilgrim & Hutchison, 1994). For example, perinatal androgen treatment increases CA3 pyramidal cell layer volume and neuronal soma size, neuronal dendritic length, the number of dendritic branches, and the overall volume of the CA3 region (Isgor & Sengelaub, 1998; Isgor & Sengelaub, 2003; Forgie & Kolb, 2003).

Recently, it has been reported that women treated with anastrozole as an endocrine therapy for breast cancer had low performance in a battery of

neuropsychological tests addressing several cognitive functions (Lejbak, Vrbancic, & Crossley, 2010; Collins, Mackenzie, Stewart, Bielajew, & Verma, 2009). Conversely, other studies found no cognitive impairment in women receiving anastrozole during 24 months (Jenkins et al., 2008). In fact, a single study performed in adult rats showed that acute anastrozole treatment dose-dependently improved spatial learning and memory tested in a water maze (Moradpour, Naghdi, & Fathollahi, 2006). Moreover, administration of a similar aromatase inhibitor (letrozole) to adult female rats also improved spatial memory (Aydin et al., 2008). Accordingly, aromatase inhibition improved working memory in male rats too (Alejandre-Gomez, Garcia-Segura, & Gonzalez-Burgos, 2007). Conversely, impaired spatial memory has been reported in aromatase-deficient knock-out mice (Martin, Jones, Simpson, & van den Buuse, 2003). Therefore, the effects of aromatase inhibition on spatial memory are still a matter of debate with conflicting results.

On the other hand, glial cells are not only a target for sex hormones, but also participate in the metabolism of these hormones, the synthesis of neurosteroids, and the modulation of neuronal activity induced by these hormones. In addition to the classical role ascribed to astroglia, namely the regulation of neural metabolism and activity, astrocytes are involved indirectly in synaptic activity by regulating the extracellular ion concentration (Sykova, 2001; Theodosis, 2002). It has also been reported that fluctuations in gonadal hormones alter hippocampal excitability and the physiology of neuron processes (Garcia-Segura, Naftolin, Hutchison, Azcoitia, & Chowen, 1999; Smith, Jones, & Wilson, 2002).

Traditionally, neurons have been specifically studied as the target of sex hormones and neurosteroids although glial cells also express receptors for these steroids (Garcia-Segura, Chowen, Dueñas, Parducz, & Naftolin, 1996). Astrocytes do not express aromatase in the brain under normal conditions, only reactive astrocytes express it after brain injury (Garcia-Segura, Naftolin et al., 1999). However, the expression of certain astroglial markers, such as intermediate filament glial fibrillary acidic protein (GFAP), is modified according to the levels of particular gonadal hormones during early development and the adult stage (Chowen, Busiguina, & Garcia-Segura, 1995; Del Cerro, Garcia-Estrada, & Garcia-Segura, 1995; Mong & McCarthy, 1999). For instance, castration of newborn males and testosterone administration to newborn females result in significant changes in GFAP expression in the hippocampus and hypothalamus (Day et al., 1990; Garcia-Segura, Chowen et al., 1996). We previously reported that perinatal

treatment with an androgen receptor blocker changed the expression of glial fibrillary acidic protein (GFAP) in the hippocampus of prepubescent rats (Conejo et al., 2005). The aim of the present study was to evaluate the expression of GFAP in the CA3 hippocampal subfield in prepubescent males and females perinatally treated with anastrozole. In addition, spatial working memory was evaluated using the Morris water maze in anastrozole-treated animals. Untreated control groups as well as vehicle-treated sex-matched groups were also included in this study.

Method

Subjects

Female and male Wistar rats from the University of Oviedo central vivarium were used. Pregnant dams received daily subcutaneous injections of 50 mg/kg/day body weight of a selective aromatase inhibitor, anastrozole (Arimidex®, AstraZeneca, Madrid, Spain) suspended in a vehicle solution containing 0.5% Tween-80 from day 17 of gestation until pups were born. This period was selected for anastrozole administration because it represents a critical period for the development of limbic brain regions (MacLusky & Naftolin, 1981; Gorski, 1989). Another group of pregnant dams was s.c. injected with the vehicle solution during the same gestational period and a third group of pregnant dams was briefly handled daily during the same period. Eight male and eight female pups from each one of the three groups of dams were assigned to the different experimental groups. Pups from the anastrozole-treated dams received daily s.c. injections of anastrozole during 18 days (5 mg/kg/day body weight suspended in the same vehicle). For injections, each litter was removed from the dam at once and was returned to their home cage in less than 12 min. A similar administration procedure was performed with pups born from vehicle-treated dams (‘pseudo’ group). Finally, pups born from the untreated dams were handled daily for 12 min during the same postnatal period (‘control’ group). After weaning at 21 days, rats were housed in standard plastic cages (27 x 27 x 15 cm) and kept on a 12:12 h light:dark cycle (lights on at 8:00 a.m.) in a temperature (21±2º C) controlled room. Food and water were available *ad libitum* throughout the course of all experiments. Animals were kept undisturbed until 29 days of age, when behavioural tests were performed. All experimental procedures were done according to the European Communities Council Directive of 24 November 1986

(86/609/EEC) and the Spanish legislation (R.D. 1201/2005) on care and use of experimental animals.

Apparatus

A black fibreglass pool measuring 1.5 m in diameter by 75 cm in height and placed 50 cm above the floor level was used as a water maze. The pool was filled with tap water to a height of 32 cm and a black escape platform was placed 2 cm beneath the water surface. The water temperature was kept at 23 ± 1 ºC during the entire test period. The experimental room had numerous visual cues such as maps, posters and plastic dishes fixed on the walls, a shelf, covered windows and a table. The experimental room was illuminated by two halogen spotlights (500 W) placed on the floor and facing the walls. Each trial was recorded and path of the animals analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

Behavioural procedure

29-day-old rats from the different experimental groups were handled and evaluated in a neurological assessment battery to discard possible motor and sensory deficits. The following day, the rats were trained in the water maze. During habituation day, rats were placed gently in the water facing the pool walls using different cardinal start points selected in a pseudorandom order, with an intertrial interval (ITI) of 30 s. A cued escape platform (painted white and placed 2 cm above the water level) was located in the centre of the pool during four consecutive trials. Rats were allowed to swim until the escape platform was found or guided there by the experimenter's hand after 60 s had elapsed, remaining on the platform during 15 s and subsequently placed in a black plastic bucket for 30s.

Animals received a single two-trial session during the second day. On the first trial, rats were released randomly from one of four start locations, and allowed to search for the visible platform located in a quadrant. The platform was also located in the same quadrant during the second trial, but rats were then allowed to search for a hidden black escape platform beneath the water surface. The rats were required to navigate to the hidden platform using spatial cues available in the room. The escape latencies were monitored and analyzed later using the video-tracking system.

Immunocytochemistry and Nissl Staining

After training, all animals from the different experimental groups were deeply anesthetized with sodium pentobarbital (70 mg/kg i.p. for males and 45 mg/kg i.p. for females) and transcardially perfused through the left cardiac ventricle with 0.9% saline in 0.1 M phosphate buffer (PBS; pH 7.4) followed by 10% phosphate-buffered formalin for 20 min. Brains were removed after perfusion and postfixed for at least 1 week in the same fixative. Subsequently, the hippocampal formation was dissected out, dehydrated with graded series of ethanol solutions (80, 96, and 100%), and embedded in paraffin. Coronal sections of the dorsal hippocampal formation contained in each paraffin block were serially cut at 20 µm with a rotary microtome (Leica; Wetzlar, Germany), selecting one of five sections on gelatinized slides. A series of alternate sections were Nissl stained with a 0.5% cresyl violet solution to enable easy discrimination of the hippocampal regions studied. The remaining sections were processed for GFAP immunocytochemistry. Briefly, after removing paraffin with toluene, sections were re-hydrated using descending concentrations of ethanol in distilled water and treated for 5 min with Tris-buffered saline (TBS) containing 0.1% Triton X-100 (Sigma; Barcelona, Spain). Sections were incu-bated for 30 min at room temperature in 1% human serum dissolved in TBS to suppress background staining. After a brief TBS rinse, sections were incubated at 4°C for 24 hr with a 1:800 dilution of rabbit anti-GFAP polyclonal antibody (Dako; Glostrup, Denmark). Excess unbound primary antibody was removed with 3 x 5 min TBS-Triton baths, and sections were incubated thereafter for 30 min at room temperature with a 1:30 dilution of biotinylated goat anti-rabbit antibody (Pierce, Rockford, USA) in 0.25% bovine serum dissolved in TBS. The tissue sections were rinsed again in TBS-Triton and incubated for 30 min in avidin-horseradish peroxidase solution (Vectastain Ultrasensitive ABC kit; Vector Labs, Burlingame, USA). After rinsing sections in TBS-Triton twice and in TBS, visualization of peroxidase enzyme activity was done by immersing sections for 30 min in a 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.01% hydrogen peroxide. Finally, sections were dehydrated with an ascending alcohol series, immersed in xylene, and coverslipped with Entellan (Merck; Darmstadt, Germany).

Stereological Quantification of Cell Counts

Stained slides were visualized using a binocular micro-scope (Olympus BH, Japan) equipped with a digital z-axis gauge (Heidenhain MT-12 microcator; Heidenhain, Traunreut, Germany) and connected via a high-resolution video camera to a black-and-white video monitor. Four square-shaped counting frames (frame size 0.025 x 0.025 mm) were drawn on the screen of the monitor. The optical fractionator stereological technique (West, 1993; West, Slomianka, & Gundersen, 1991) was applied to esti-mate total cell counts in selected hippocampal regions. Briefly, a series of equidistant sections (eight sections on average) selected in a systematic uniform random manner from all sections com-prising the hippocampus were placed under a 100X oil immersion lens. In each section, the microscopic fields viewed on the video monitor, including the regions of interest and the hip-pocampal regions, were sampled systematically according to an x- and y-axis microscope stage movement sequence with a certain step. Additionally, only cells within the counting frame and focused below 2.5 µm (guard height) and above or equal to 15 µm in the z-axis direction were counted. The sampled tissue area (XY area) can be estimated by taking into account the total tissue area covered by all counting frames used (0.16 mm^2 on average for each hippocampal area), and the fraction of sections comprising the hippocampal regions of interest (1/12). The number of cells (N) of the sampled fraction from a particular brain region can therefore be estimated using the following formula:

$$N = (\sum Q^{-}) \times (1/12) \times (XY\ area/a) \times (t/h)$$

where $\sum Q^{-}$ is the number of cells counted in the selected sections, a represents the area of the tissue covered by each counting frame (0.025 x 0.025 mm), and h the height of the total section thickness (t) sampled in the z-axis direction (h or optical disector height: 15 µm). The estimate of the total GFAP-positive cells in the selected hippocampal regions, a measure of the total volume (V) of these regions, can be carried out by applying the Cavalieri principle (Gundersen et al., 1988). Using a microscope equipped with a drawing tube, the anatomic boundaries of the hippocampal regions selected in each section used for the optical fractionator procedure were drawn on a paper. The number of points included within the boundaries of the CA3 region profile was calculated by randomly superimposing a transparent point grid on the images drawn on the paper. An unbiased estimate of the volume of each region is given by T x

$\sum$ P x a(p), where T is the thickness of the brain region selected, derived from the total number of sections obtained from it (100 sections on average) and the thickness of each section (20 µm), $\sum$P is the total number of points counted for each region, and a(p) is the area associated with each point of the grid (20.25 mm^2) corrected for the microscopic magnification used (55X). Finally, the total number of cells can be obtained by multiplying V x N. The total number of GFAP-positive cells (GFAP-ir) was estimated in the dorsal CA3 area (collecting the GFAP-ir cells found mainly in the stratum oriens, stratum radiatum, and stratum lacunosum-moleculare). The anatomic boundaries were based on the atlas of Paxinos and Watson (1986), using the Nissl-stained sections as a reference for the GFAP-stained alternate sections. In sections where the hippocampus extended all along its dorsoventral axis, the limit between dorsal and ventral regions corresponded to the dorsoventral coordinate -5.0 mm from bregma according to Moser, Moser, Forrest, Andersen, and Morris (1995).

Data analysis

Behavioral data. Differences in mean escape latencies between the sample and retention trials in each group were analyzed using paired t-tests; p values less than 5% were considered as statistically significant.

Stereological quantification. Differences in the number of GFAP-positive astrocytes between experimental groups for each sex were analyzed by one-way analysis of variance (ANOVA). Tukey's HSD tests were used to evaluate differences between training days where appropriate. The coefficients of error and variation for each estimate of the total number of GFAP-positive astrocytes in each hippocampal area were calculated for all experimental groups according to Gundersen and Jensen (1987).

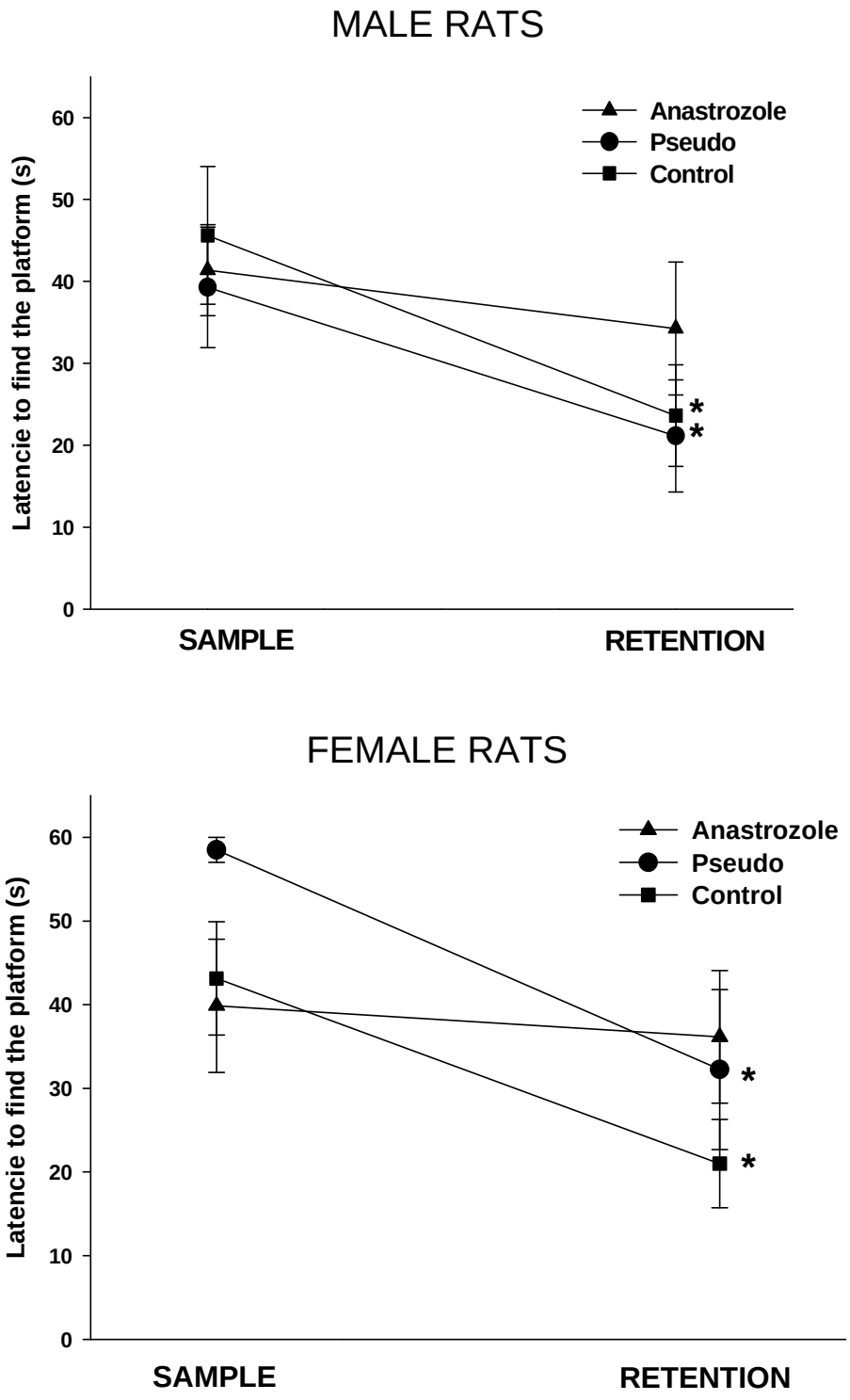
Results

Behavioral results

In the female anastrozole-treated groups, a lower escape latency was found in the retention trial as compared with the sample trial in the control, $t(7)= 2.84$; $p \leq .05$, and pseudo, $t(7)= 3.22$; $p \leq .01$, groups. Likewise, the control and pseudo male experimental groups had a significantly lower escape latency in the retention trial as compared with the sample trial, $t(7)= 2.54$; $p \leq .05$ and $t(7)= 2.53$; $p \leq .05$, respectively.

No differences were found between sample and retention trial in anastrozole groups of both sexes (see Figure 1).

Figure 1. Mean escape latencies (±S.E.M.) measured in the different experimental groups. **A**. male groups **B**. female groups. *significantly different as compared to the sample phase ($p < .05$, Tukey's post-hoc test).



Number of GFAP-ir Astrocytes

Table 1 shows that the number of GFAP-ir astrocytes in 30-day-old animals was significantly different according to the treatment in female rats, $F(2,17)= 5.28$; $p \leq .05$, and male rats, $F(2,17) = 20.82$; $p \leq .001$). Post-hoc analysis revealed that the number of GFAP-ir cells in CA3 hippocampal area was higher in the pseudo group as compared to the control group only in female rats ($p \leq .05$). No differences were found between the anastrozole female group as compared to the other female experimental groups. As regards to the male experimental groups, we found a lower number GFAP-ir cells in CA3 hippocampal area in the anastrozole group as compared to control ($p \leq .001$) and pseudo ($p \leq .001$) groups. The typical morphology of immature GFAP-ir astrocytes is shown in Figure 2.

Table 1. Total number of GFAP-ir astrocytes (mean±SEM) found in the CA3 dorsal hippocampal region in male and female rats of the three experimental groups.

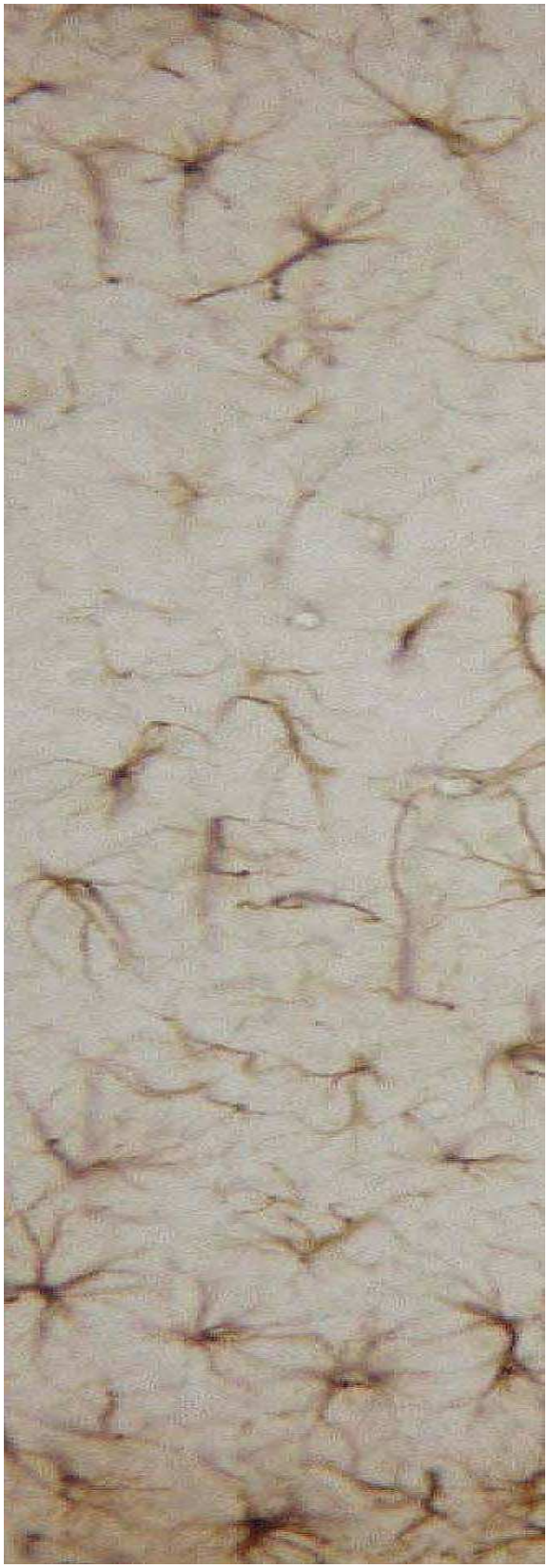
	ANASTROZOLE	**PSEUDO**	**CONTROL**
MALES	23,176.0 ± 1,792.3*	31,939.1 ± 836.6	32,542.8 ± 215.6
FEMALES	25,120.3 ± 1,401.5	27,396.0 ± 272.6	23,610.0 ± 148.4†

Note. * $p < .05$ as compared to the rest of groups, $^{\dagger}p < .05$ vs. PSEUDO group.

Discussion

Control male and female groups performed similarly in the spatial working memory task as previously reported in both prepubescent (Conejo, González-Pardo, Vallejo, & Arias, 2004) and adult rats (Healy, Braham, & Braithwaite, 1999). Apparently, it seems that the gestational and perinatal organizational effects gonadal or sex hormones on the brain did not affect spatial working memory performance. However, perinatal inhibition of aromatase by finasteride clearly impaired spatial working memory both in male and female rats. As previously reported, aromatase-deficient knock-out mice displayed impaired spatial memory (Martin et al., 2003) although subchronic administration of aromatase inhibitors actually improved spatial working memory in male and female rats (Aydin et al., 2008; Alejandre-Gomez et al., 2007).

Figure 2. Microphotograph showing the morphology of GFAP-positive astrocytes in the rat hippocampus at 30 days of age.



As hypothesized, aromatase inhibition in males prevented testosterone to masculinise their brains explaining the absence of sex differences in the spatial working memory task. However, an unexpected result was the working memory impairment in female rats. In fact, there is evidence reporting that adult women receiving anastrozole had mild cognitive deficits including memory impairment in several studies (Lejbak et al., 2010; Collins et al., 2009; Bender et al., 2007). It is know that aromatase inhibition in female rats particularly decreases hippocampal catecholamine content (Aydin et al., 2008) and induces synaptic loss in the hippocampus in ovariectomized mice (Zhou et al., 2010). Hippocampal function may be thus impaired by aromatase inhibition with anastrozole in female rats and it would also affect males by hindering the action of estrogens derived from testosterone in male brain. Accordingly, the hippocampus is related with spatial working memory (Bird & Burgess, 2008) and specifically the dorsal CA3 region (Kesner, 2007; Gilbert & Kesner, 2006). Therefore, spatial working memory deficits in male and female rats may be at least in part explained by the specific actions of aromatase in the developing hippocampus of male and female rats. An alternative explanation would be based on the role of aromatase on the synthesis of neurosteroids together with estrogens in the hippocampus (Boon, Chow, & Simpson, 2010; Rune & Frotscher, 2005). Neurosteroids and estrogens have neuroprotective actions on the brain and promote cell survival and exert complex actions on brain function (Boon et al., 2010).

On the other hand, treatment with finasteride decreased the number of GFAP-positive astrocytes in the dorsal CA3 hippocampus in males but not females. Moreover, control males had higher numbers of GFAP-positive astrocytes than control females in this hippocampal area in agreement with our previous study (Conejo et al., 2005). Although aromatase is not normally expressed in glial cells, aromatase inhibition would indirectly affect the survival and proliferation of astrocytes by decreasing the level of estrogens in the hippocampus. Estrogen receptors are found in astrocytes that modulate neuronal synaptic plasticity involved in turn in learning and memory (Azcoitia, Santos-Galindo, Arevalo, & Garcia-Segura, 2010). In addition, it is known that sex hormones affect the expression of GFAP in male rat hippocampus (Day et al., 1993). Accordingly, we found previously that GFAP expression in the dorsal hippocampus critically depended on early exposure to gonadal hormones like testosterone in particular (Conejo et al., 2005). It would be thus possible that aromatase inhibition would specifically affect the expression of GFAP in male hippocampus given that the conversion of

testosterone to estradiol acting in astrocytes was suppressed. Although spatial working memory was also impaired in females treated with anastrozole, the underlying mechanisms would be probably not related with astrocyte function but more with neuronal plasticity and metabolism.

Lastly, females treated with vehicle solution had significantly different number of GFAP-positive astrocytes as compared with control group. Although it is certainly difficult to elucidate the specific mechanisms involved in the sex-specific effects of treatment with the vehicle solution, stress derived from daily injections during both prenatal and postnatal periods would play an important role. It is known that stress during the early postnatal development (especially during 4-14 postnatal days) affects the development of the hypothalamic-pituitary-adrenal (HPA) axis related with the physiological mechanisms of the stress response (Levine, 2002). Glucocorticoids are hormones involved in the stress response affecting cell survival and proliferation together with GFAP expression particularly in the hippocampus (Nichols, Agolley, Zieba, & Bye, 2005). In addition, sex-specific effects of prenatal stress on neuroendocrine responses have been reported in rats (Brunton & Russell, 2010) that could explain the different effects of stress on GFAP expression in the hippocampus. In this regard, female rats are more sensitive than males to prenatal stress shown by increased anxiety and HPA axis response to stress (Weinstock, 2007).

In summary, aromatase inhibition during the perinatal period caused sex-specific changes in GFAP-positive astrocytes in the CA3 region of the dorsal hippocampus and impaired spatial working memory in male and female prepubescent rats. These effects would be mediated by complex mechanisms involving a neuroprotective role of estrogens on hippocampal development in both sexes. Additional studies are required to gain further knowledge about the key role of aromatase on brain development and behaviour.

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