Sexual dimorphic stages affect both proliferation and serotonergic innervation in the adult rostral migratory stream

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ABSTRACT

One of the sexual dimorphic differences in adult rodents is neural proliferation. Here we demonstrate that physiological hormone stages can modulate this proliferation in the adult forebrain. Female mice, both pregnant and synchronized in oestrus, exhibited higher proliferating cell percentages than males in both the rostral migratory stream (RMS) and the olfactory bulb (OB). Moreover, although the hormonal component also influenced the subventricular zone (SVZ), no differences in proliferation were observed in this region. In addition, both groups of females had higher numbers of serotonergic fibres in these regions. Serotonin may therefore be related to the mechanism of action by which hormones can affect cell proliferation of this brain region. We also evaluated cell death in the SVZ in males and females, finding that this was higher in the former. Taken together, our results support the idea that in female rodents more neuroblasts are able to reach the RMS and then proliferate, apoptosis being an additional mechanism affecting the low proliferation of cells in the RMS and OB in males. Thus, proliferation in the RMS is influenced by sexual dimorphism.

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Introduction

Neurogenesis in the adult mammalian forebrain is a process restricted to two regions: the hippocampus (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1997; van Praag et al., 2002) and the subventricular zone (SVZ; Altman, 1969; Carleton et al., 2003; González-Granero et al., 2006). The SVZ is a remnant of the embryonic ventricular zone and is mitotically active throughout the lives of rodents (Altman, 1962; Doetsch et al., 1997). Neuroblasts arise from the SVZ and migrate tangentially along a pathway called the rostral migratory stream (RMS) until they reach the olfactory bulb (OB; Altman, 1969; Carleton et al., 2003; Lois and Álvarez-Buylla, 1994). This tangential migration is supported at least by astrocytes forming the so-called "glial tubes" (Doetsch et al., 1997; Lois et al., 1996; Peretto et al., 1997). Once in the OB, neuroblasts migrate radially from the extension of the RMS in the OB (RMS-OB) to outer layers. Finally,

neuroblasts differentiate into interneurons (Altman, 1969; Carleton et al., 2003; Lois and Álvarez-Buylla, 1994).

Several factors affect the proliferation, migration, differentiation and survival of the newly formed neuroblasts: i.e., growth factors (Doetsch et al., 2002; Jin et al., 2001; Jin et al., 2002; Kirschenbaum and Goldman, 1995; Shingo et al., 2001; Tropepe et al., 1997; Zigova et al., 1998), cell adhesion molecules (Belvindrah et al., 2002; Capela and Temple, 2002; Conover et al., 2000), morphogens (Chambers et al., 2001; Lim et al., 2000), neurotransmitters (Banasr et al., 2001; Banasr et al., 2004; Hoglinger et al., 2004; Packer et al., 2003) and hormones (Banasr et al., 2001; Giachino et al., 2004; Scharfman and Maclusky, 2005; Shingo et al., 2003; Smith et al., 2001).

Among the sexual hormones, the female counterparts exert a strong effect upon the SVZ. For example, prolactin has been described to increase neural proliferation in the SVZ of pregnant mice (Shingo et al., 2003). By contrast, progesterone has no effect on that process, but its derived products dihydroprogesterone (DHP) and tetrahydro-progesterone (THP) diminish the proliferation rate (Giachino et al., 2004). Estrogens seem to have no effect on neuroblast proliferation in the SVZ of adult mice (Hoyk et al., 2006; Shingo et al., 2003) but do exert a positive effect on the SVZ of other rodents (Smith et al., 2001). Estrogens also promote neurogenesis in the hippocampus (Mazzucco et al., 2006; Tanapat et al., 1999). Additionally, most experiments with hormones have used non-physiological concentrations, which may elicit an effect contrary to the physiological one (Mazzucco et al.,

2006). Moreover, in certain pathological situations such as stroke, normal and constant levels of estrogens (not single acute injections) induce an increase in cell proliferation in the SVZ (Suzuki et al., 2007).

At least in the hippocampal dentate gyrus, an increase in newly generated neuroblasts (via an indirect signaling pathway induced by estrogens) has been demonstrated (Banasr et al., 2001). Estrogens can act directly on neuroblasts through specific receptors, but their effect can also be mediated by other molecules, such as serotonin (Banasr et al., 2001). Indeed, serotonin has been described to be a stimulant of neural proliferation in both the SVZ and in the hippocampus (Banasr et al., 2004).

The aim of the present work was to determine whether different sexual dimorphic stages in adult mice might influence neural proliferation in the mouse SVZ and in its extension, the RMS. We chose physiologic stages, without gonadal ablations or direct injections of female oestral hormones (such as estrogens or progesterone). We also analyzed the density of serotonergic fibres in the different regions of interest and at their periphery. Finally, we evaluated cell death in the areas studied.

Materials

100-day-old mice of the C57BL/6J strain (*Mus musculus*, L. 1758) were used. The animals were housed at the Animal Facilities of the University of Salamanca at constant temperature and humidity, with a 12/12 hour photoperiod, and were fed "ad libitum" with water and special rodent chow (Rodent toxicology diet, B&K Universal G.J., S.L. Molins de Rei, Barcelona). Four groups of animals were used: control males (7 animals), hormone-injected males (6 animals), oestrus-synchronized females (7 animals) and pregnant females (6 animals).

All animals were housed, manipulated and sacrificed in accordance with current European (86/609/ECC) and Spanish legislation (RD 1201/2005).

Hormonal standardization

All female mice were injected intraperitoneally with two hormones to induce them to ovulate and to synchronize their oestral cycle: pregnant mare serum gonadotropin (PMSG; Foligon, Intervet Laboratories S.A., Salamanca, Spain) and human chorionic gonadotropin (HCG; Veterin Corion, Divasa-Farmavic S.A., Barcelona, Spain). First, 100 μl of PMSG was injected at a concentration of 0.125% (v/v) in saline serum and 48 h later HCG was injected at the same volume and concentration. Both hormones were injected at 1:30 pm (Nagy et al., 2003). This cocktail of hormones has been reported to be an excellent mechanism for inducing ovulation in rodents by increasing both follicle stimulating and luteinizing hormones without other side effects (Fowler and Edwards, 1957; Kon et al., 2005). Notwithstanding, one group of males was injected with the same gonadotropins to assess possible side effects (in proliferation, the density in serotonergic fibres, or cell death) in the five rostro-caudal brain areas of interest, as defined below, in the "Analysis" section. The synchronic females and the hormone-injected males were sacrificed at 09:00 on the morning after the second injection. Following the second injection, the other group of females (pregnant) were kept overnight with a male for conception to take place. This group was sacrificed 12 days later in order to avoid the effect of prolactin on cell proliferation (Shingo et al., 2003).

Tissue preparation

The animals were deeply anaesthetized with 1 μl/g (body weight) of a mixture containing ketamine hydrochloride (Ketolar; Parke-Davis, Barcelona, Spain) and xylazine hydrochloride (Rompun; Bayer, Leverkussen, Germany) at a proportion 3:4. Then, they were perfused intracardially with 0.9% NaCl, followed by 5 ml/g b.w. of Bouin-4%

fixative (4% w/v of depolymerized paraformaldehyde, 75% v/v saturated picric acid and 5% v/v acetic acid (Valero et al., 2005). After perfusion, the brains were dissected and post-fixed with the same solution for 2 h. Brains were rinsed for 2 h with 0.1 M phosphate buffer, pH 7.4 (PB).

Tissue blocks were cryoprotected by immersing them in 30% (w/v) sucrose in PB. When the blocks had sunk, they were frozen with liquid nitrogen and stored at −80 °C until sectioning. 40-μm thick slices were obtained using a freezing-sliding microtome attached to a freezing unit. Sections were rinsed in 0.1 M PB (3 × 10 min), and then stored at −20 °C in a freezing mixture made of 30% (v/v) glycerol and 30% (v/v) polyethyleneglycol in phosphate buffer, pH 7.4.

Immunofluorescence

Freeze-stored sections were warmed to room temperature and then thoroughly rinsed for 5 × 10 min with PB. Then, they were incubated with 0.13 M sodium borohydride for 20 min to eliminate aldehyde autofluorescence (Weruaga-Prieto et al., 1996). Tissue slices were washed in phosphate buffered saline (PBS; 3 × 10 min) and incubated for 48 h at 4 °C under continuous rotary shaking in a medium containing PBS, Triton X-100 at 0.2% (w/v), normal goat serum at 5% (v/v), and the primary antibody. Following this, the sections were washed in PBS (3 × 10 min) to remove excess antibody and were then incubated in a second medium for 2 h at room temperature under continuous rotary shaking. This second medium contained PBS, Triton X-100 at 0.2% (w/v) and the secondary fluorescent antibody. Thirty minutes before the end of the incubation, propidium iodide (PI; Sigma-Aldrich, St Louis, EEUU) 1:2000 v/v was added to the medium in order to obtain a counterstain.

To detect proliferating cells, the proliferating cell nuclear antigen (PCNA) immunolabelling technique was employed (Santa Cruz Biotechnology, Santa Cruz, California, EEUU; 1:3000 v/v). To detect serotonergic fibres, anti-serotonin transporter polyclonal rabbit IgG (kindly provided by Dr. Zhou, Anatomy Department, Indiana University School of Medicine, EEUU; 1:10000 v/v; Zhou et al., 1996) was chosen. The serotonin transporter is localized in all the nervous fibres that use serotonin to reuptake the neurotransmitter (Zhou et al., 1996). In addition, this staining avoids the problems involved in the direct staining of serotonin, such as variations in the levels of this neurotransmitter. In this sense, the accumulation of serotonin in the fibre terminals can increase the immunostaining with no increase in innervation (Huether et al., 1997; Zhou et al., 1996). In order to check the phenotype of proliferating cells, double immunofluorescence was performed with anti-PCNA and specific cellular markers: anti-glial fibrillary acidic protein (GFAP) polyclonal rabbit IgG (Sigma, Missouri, EEUU; 1:1000 v/v) to label astrocytes and anti-β-tubulin III (Tuj1) polyclonal rabbit IgG (Covance, California, EEUU; 1:1000 v/v) to label neuroblasts. Specific secondary anti-goat IgG bound to Cy2 or Cy5 fluorescent probes was used.

Finally, the sections were rinsed in the darkness in PBS and mounted with coverslips using a freshly prepared anti-fading medium containing 0.42% (w/v) glycine, 0.021% (w/v) sodium hydroxide, 0.51% (w/v) sodium chloride, 5% (w/v) N-propyl-gallate, 70% (v/v) glycerol and 0.002% (w/v) Thymerosal in distilled water.

TUNEL technique

To detect apoptotic cells, the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labelling) technique was employed. This procedure stains free OH-3′ DNA endings formed during endonuclease-programmed cell death events.

Tissue slices were washed with PBS (3 × 10 min) and incubated for 20 min in sodium borohydride solution (0.5% w/v) in PBS. They were then washed with PBS (3 × 10 min) and permeabilized for 15 min with Triton X-100 0.2% (v/v) and sodium citrate 0.1% (p/v) in distilled water.

Following this they were washed again with PBS (2×10 min) and incubated with TUNEL buffer for 30 min. This buffer comprised 30 mM Tris–HCl, 140 mM sodium cacodylate, 1 mM CoCl and 0.3% Triton X-100 (v/v). Then, the tissue slices were incubated in a medium with terminal transferase and biotinated dUTP in TUNEL buffer for 2 h. The reaction was terminated by the addition of saline sodium citrate buffer (0.15 M NaCl and 0.015 M sodium citrate). Finally, the slices were washed again with PBS (3×10 min) and developed with a medium containing the Cy2 fluorescent probe attached to a streptavidin molecule, which permits its binding to the biotinated dUTP, diluted in PBS. Both PI staining and slide mounting were performed similarly to the procedures used in the immunofluorescence technique.

Sudan Black staining

After the immunofluorescence or TUNEL techniques, and once the tissue slices had been mounted on microscope slides, the tissue was stained with Sudan Black to mask the background fluorescence due to lipids, especially those present in myelin. This staining does not mask specific fluorescence staining (Viegas et al., 2007). The slides were rinsed in 70° (v/v) ethanol, after which they were stained with a 1% (w/v) Sudan Black solution in 70° (v/v) ethanol for 3 min. Following this, they were rinsed again in 70° (v/v) ethanol and washed in PB 0.1 M (5×10 min).

Analysis

Three large proliferating regions were analyzed: SVZ, RMS and RMS-OB. For a better comparison of the experimental groups, five equidistant and comparative rostro-caudal levels were selected as previously described (Weruaga et al., 1999), designated SVZ I (Bregma: 0.5 mm), SVZ II (Bregma: 1.3 mm), RMS I (Bregma: 2.1 mm), RMS II (Bregma: 2.9 mm) and RMS-OB (Bregma: 3.7 mm) (Hof et al., 2000). The regions of interest in each level were those showing proliferation: the SVZ with its dorsal horn, the RMS itself, and the extension of the RMS in the middle of the OB.

To determine neural proliferation, the PCNA-positive cells in the zones of interest of each level were counted unilaterally in focal planes. As expected, we did not observe differences between either brain hemisphere. Accordingly, the side of each section was chosen at random. The percentages of PCNA-positive cells in these areas were calculated, referring these to the total numbers (labelled with PI).

In order to determine the density of serotonergic fibres, the focal planes of the regions of interest in each level studied were scanned. Using the Image J program (version 1.37, Wayne Rasband, National Institutes of Health, USA), the images were converted into 8-bit format (black and white) to quantify the number of pixels of the serotonergic fibres. The pixels were also quantified in the peripheral areas of the regions of interest. Thus at the level of the RMS and OB (RMS I, RMS II and RMS-OB), the peripheral fibres were quantified at a ratio of 50 μm; in the levels of the SVZ (SVZ I and SVZ II), the peripheral fibres were analyzed only in the adjacent striatum, located close to the ventro-lateral region of the SVZ, since this was the only region where serotonergic fibres were visualized in these levels. Thus, a pixel density of the serotonergic fibres in the regions of interest and in their peripheral areas was established.

The density of apoptotic cells was very low, and hence all the TUNEL-stained cells were counted and the areas of interest were measured in all slices; not only in the five representative levels. In this way, the density of apoptotic cells in the three large proliferating regions – SVZ, RMS and RMS-OB – was obtained as a whole.

All counts were performed by the same person, following the same criterion and using a double blind study.

Once homoscedasticity (quality of samples with normal distribution and equal variances) had been checked with the Kolmogorov–Smirnov test, the two-way ANOVA test was employed to analyze possible differences due to the "level" or "group" factors in each experiment. Following this, two different one-way ANOVA analyses were performed for each experiment in order to assess possible differences among the levels in each group or among groups in each level. Only when the Levene test yielded non-homogeneous variance among the samples was the Kruskal–Wallis test employed instead of the ANOVA test. Thereafter, and depending on the homogeneity of the variance, *post hoc* tests or the Mann–Whitney U test were applied to detect differences between experimental groups or levels.

Results

Proliferation

PCNA staining was clearly nuclear, corresponding to cells within the cell cycle (Fig. 1). In fact, some mitotic figures were detected: metaphasic or anaphasic chromosomes stained with PI in PCNA-positive nuclei (not shown). These data are in agreement with those reported previously showing that PCNA begins to be expressed at the end of the G_1 phase, increases strongly in the S phase, decreases slightly in the G_2 and M phases, and eventually disappears (Kurki et al., 1986). In addition, we observed that the proliferating cells had different degrees of PCNA staining, depending on their progression in the S phase, as we have described previously (Valero et al., 2005): early S-phase cells (with a large number of punctate spots throughout the nuclei), mid-S phase cells (with perinuclear, perinucleolar or intranucleolar rims of numerous small and discrete granules) and late S-phase cells (with large granular spots or "patches" in their nuclei).

PCNA-stained cells were restricted to the proliferating or migrating regions: i.e., the SVZ and the RMS (Figs. 1a–d; Supplementary Figs. 1a–d). However, scarce PCNA-positive nuclei were detected outside the RMS. Their presence outside this location may be due to cell migration towards the accessory olfactory bulb (Bonfanti et al., 1997).

In the analysis of proliferating cells, the two-way ANOVA test revealed differences among groups in the global percentage of proliferating cells. *Post hoc* tests showed that both groups of females had a higher percentage than the two groups of males (Fig. 2a; $p < 0.01$). On analyzing each anatomical level, one-way ANOVA demonstrated that these differences between sexes were focused in the RMS I ($p < 0.05$; Figs. 1a–d and 2 b), RMS II ($p < 0.01$; Fig. 2b) and RMS-OB ($p < 0.05$; Fig. 2 b) levels. By contrast, in the SVZ I level sexual differences were observed between the synchronized (control) females and the other three groups (Fig 2b, $p < 0.05$; Supplementary Figs. 1a–d) and in the SVZ II level hormone-treated males had less proliferation than the other animals (Fig. 2b, $p < 0.05$).

Additionally, the two-way ANOVA test also revealed global differences among the rostrocaudal levels: each level was different from the others, except for SVZ I and RMS I, which had similar percentages. In this global distribution, the SVZ II level showed the highest rate of proliferation (*post hoc* tests, $p < 0.01$). These global differences were compared within each group by one-way ANOVA. All males and pregnant females had cell proliferation distribution patterns similar to the global one, the SVZ II level having the highest percentage, and the SVZ I and RMS I levels having similar percentages. However, the synchronized females exhibited a different pattern: in this group, the SVZ I, SVZ II and RMS had the same percentage, while the other levels had lower percentages.

In short, our results demonstrated that the percentage of proliferating cells was higher in the females than in the males, and these differences among the groups were apparent throughout the RMS, including the OB.

Cell characterisation

As previously reported (Doetsch et al., 1997; Lois et al., 1996; Suzuki et al., 2007), double immunofluorescence techniques for PCNA
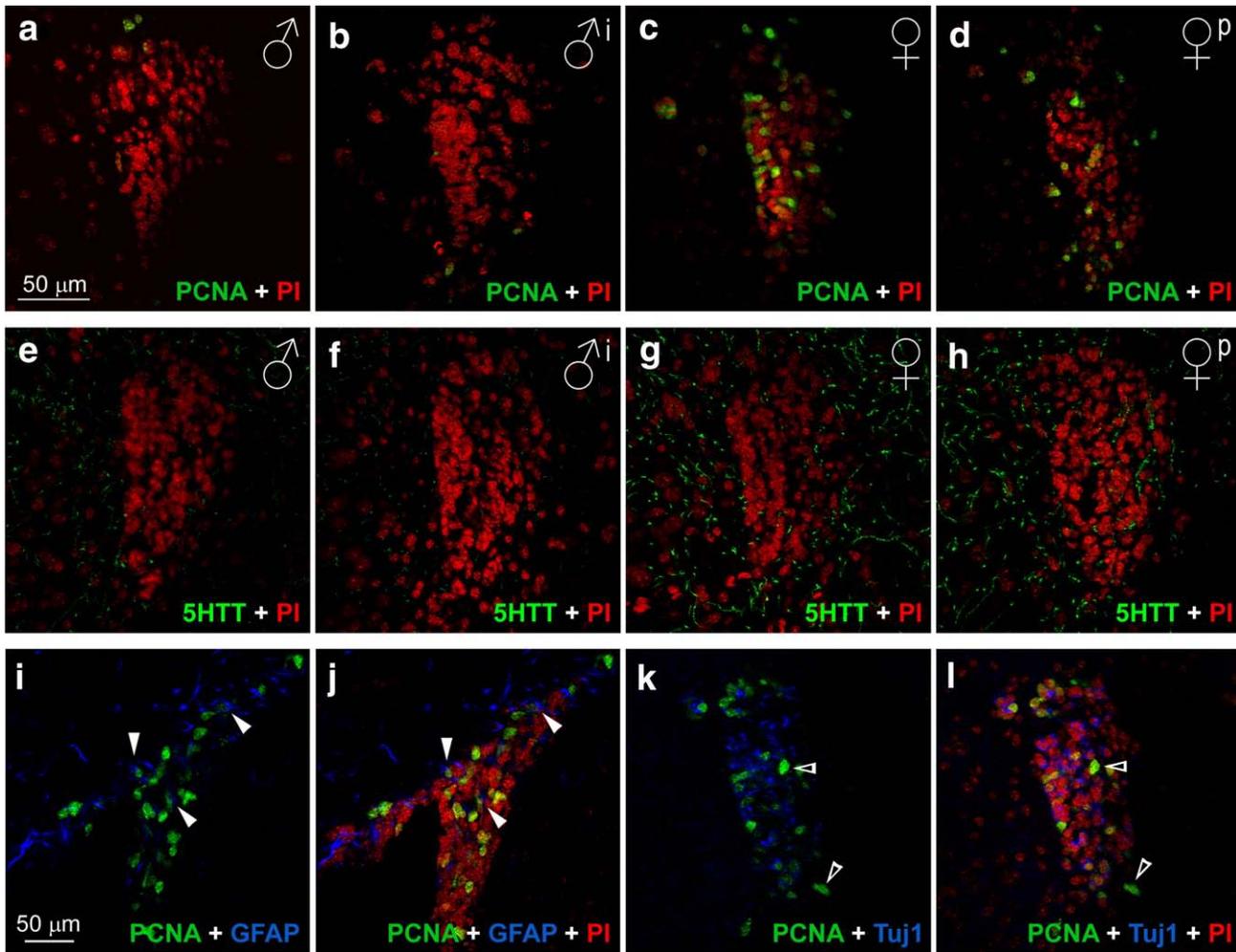
Fig. 1. Confocal images at the RMS I level (a–h). This region reflects the global results in cell proliferation (a–d), and serotonergic innervation (e–h), between sexes. In a–d proliferating cells are labelled with PCNA (green); synchronized (c) and pregnant females (d) have higher percentages of proliferating cells than control (a) and hormoned males (b). In e–h serotonergic fibres are labelled with the serotonin transporter (5HTT, green). Synchronized (g) and pregnant females (h) show a greater density of serotonergic fibres than males (e, f). All cell nuclei are stained with PI (red). Confocal images of the SVZ and RMS (i–l). Markers of both proliferating cells (PCNA, green) and astrocytes (GFAP, blue) only colocalize within the SVZ (i, j, arrowheads); the vast majority of proliferating cells are neuroblasts (k, l) colocalizing for both specific markers: PCNA (green) and Tuj1 (blue); however, some proliferating cells are not neuroblasts (k, l, open arrowheads). Cell nuclei are stained with PI (j, l, red). Photographs a–h have different scale bar to i–l.

and Tuj1 or PCNA and GFAP revealed that the majority of proliferating cells were neuroblasts (Figs. 1i–l). Some proliferating cells only exhibited astrocytic characteristics in both levels of the SVZ: SVZ I and SVZ II (Figs. 1i, j). In the RMS, some proliferating cells did not express specific neuronal markers (Figs. 3k, l). Since no proliferating astrocytes were observed in the RMS, such proliferating cells were probably neural progenitors, the so-called Type C cells (Doetsch et al., 1997; Doetsch et al., 2002; Peretto et al., 1999).
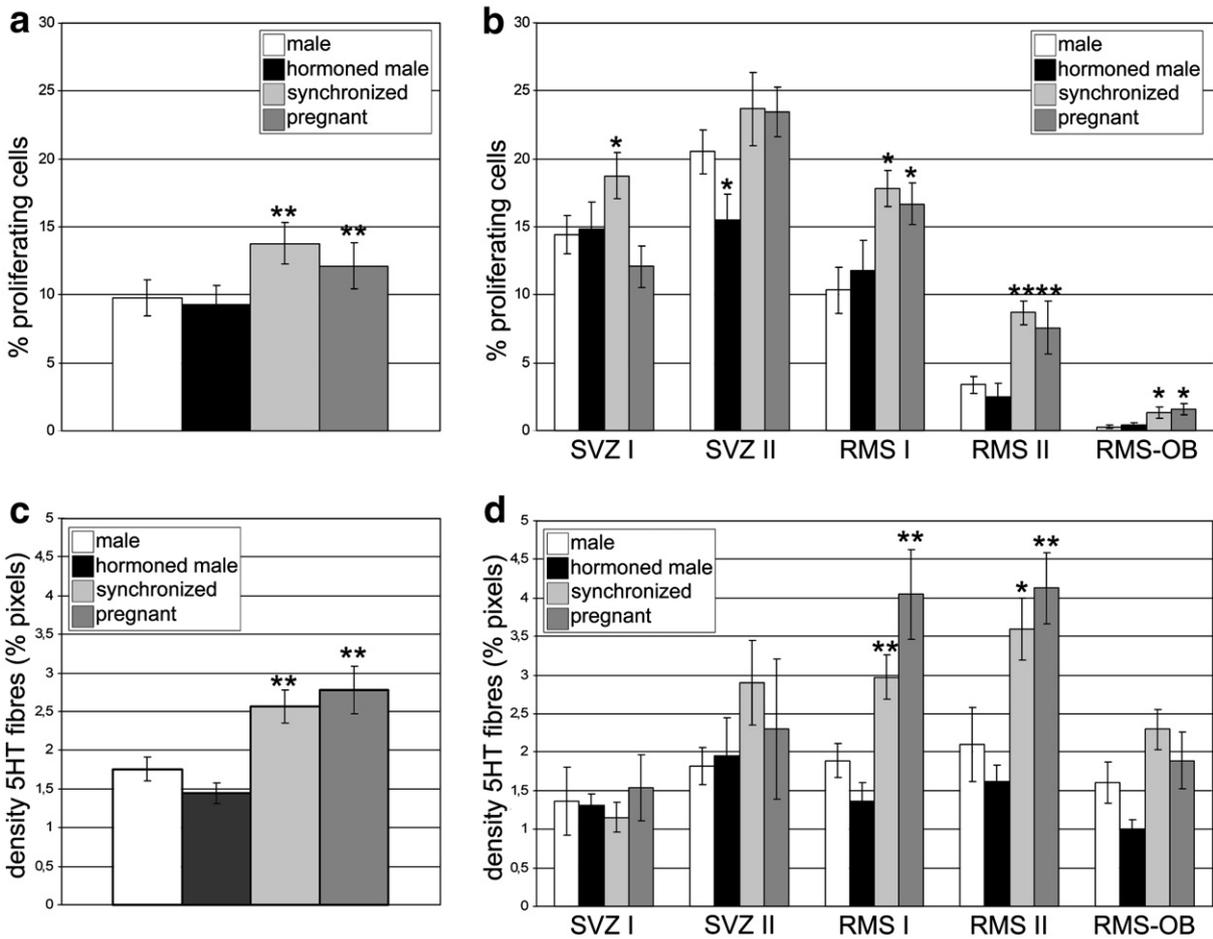
Serotonergic fibres

Serotonergic fibres were detected in the SVZ, throughout the RMS, and also around it (Figs. 1e–h; Supplementary Figs. 1 e–h). However, close to the SVZ serotonergic fibres were only observed in the adjacent striatum, but not in the corpus callosum (Supplementary Figs. 1e–h).

As explained above, we measured the density of serotonergic fibres not only in the regions of interest but also in their immediate periphery. The two-way ANOVA test revealed global differences in the density of serotonergic fibres among groups. *Post hoc* analysis confirmed that the highest percentage corresponded to both groups of females (Fig. 2c). These differences were observed both in regions of interest and at their periphery ($p < 0.01$). The one-way ANOVA test performed in each level among groups also demonstrated that the

differences were present only in the RMS levels ($p < 0.01$). In RMS I, differences were seen between the two groups of females and the males (Figs. 2d; *post hoc* analysis $p < 0.1$), with a higher density in pregnant than in synchronized females (Figs. 2d; *post hoc* analysis $p < 0.5$); in the RMS II, differences appeared between both groups of females and the groups of males (Fig. 2d; *post hoc* tests $p < 0.05$). The differences were found both in the areas of interest and at their periphery.

The two-way ANOVA test demonstrated global differences among rostrocaudal levels ($p < 0.01$). The RMS I and RMS II levels had the highest density values in both areas studied and also at their periphery as a global distribution, without separating the four different groups (*post hoc* tests $p < 0.01$). One-way ANOVA performed in each group revealed the same differences in both groups of females. Thus, RMS I and RMS II had higher densities than the other levels (*post hoc* analysis $p < 0.05$). However, there were no differences among levels in the males (*post hoc* analysis $p > 0.05$). The absence of differences among levels in both male groups is in agreement with their lower densities in the RMS as compared with females.

In sum, our results showed that the density of serotonergic fibres was higher in both groups of female mice than in the males. Specifically, we found that these differences were concentrated along the RMS, similarly to the results concerning proliferation.

Fig. 2. Charts showing the quantitative results on both cell proliferation and serotonergic inner innervation. a, Global percentage of proliferating cells; b, percentage of proliferating cells sorted by levels; c, global density of serotonergic fibres; d, density of serotonergic fibres in each level. One asterisk indicates $p < 0.05$; two indicate $p < 0.01$.

Apoptotic cells

We observed apoptotic cells labelled with the TUNEL technique in all regions studied, with differences in their fluorescence densities (as described below). The TUNEL technique and PI staining allowed us to visualize typical chromatin condensation (Supplementary Fig. 2), as previously described (Charriaut-Marlangue et al., 1996; Gavrieli et al., 1992; Sairanen et al., 2006).
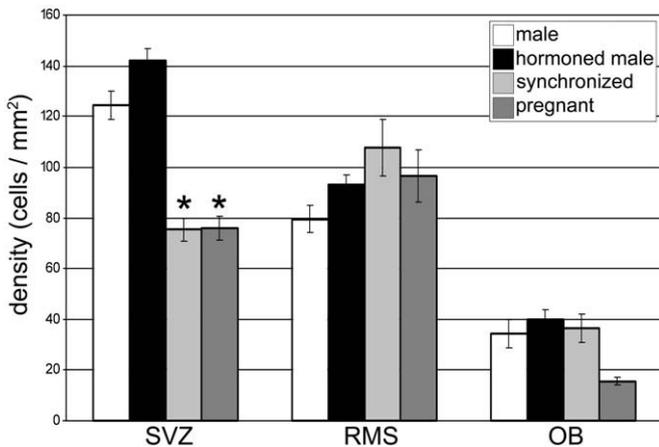
Fig. 3. Density of apoptotic cells as visualized with the TUNEL technique sorted by regions. Differences between the sexes are only seen in the SVZ, the males exhibiting higher cell death rates than females. Asterisk indicates $p < 0.05$.

The two-way ANOVA analysis revealed global differences among regions ($p < 0.01$), with a higher density of apoptotic cells in the SVZ. However, no global differences were found among groups ($p > 0.05$). The one-way ANOVA analyses performed for each group and their *post hoc* tests revealed that only the two groups of males showed differences with a higher density of apoptotic cells in the SVZ, but not females. Hence, we decided to perform one-way ANOVA analyses for each zone. This test and its *post hoc* tests revealed that only in the SVZ was the density of apoptotic cells higher in both groups of males than in females (Fig. 3; $p < 0.01$).

Accordingly, our results show that males develop more apoptotic cells than females in the SVZ.

Discussion

Our experiments revealed a higher percentage of proliferating cells in both groups of females than in males. Most of such cells were neuroblasts. In addition, a higher density of serotonergic fibres was seen in the females. These differences were centred in the region of the RMS, and a higher density of apoptotic cells was observed in the SVZ of the males than in that of the females. Between the two groups of males there were no differences in any experiment. Therefore, the injection of gonadotropins only elicited ovulation in females and had no direct effects on the neural proliferation of the regions analyzed. This statement is in agreement with previous observations (Fowler and Edwards, 1957; Kon et al., 2005).

These results suggest that sexual dimorphism exists in adult rodent neurogenesis, as reflected in a higher global percentage of

proliferating cells in the two groups of females as compared to males. These overall results were repeated in the different levels of the RMS I, RMS II and RMS-OB.

In a previous work, it was suggested that differences in neural proliferation in the SVZ between males and females are exclusively due to prolactin (Shingo et al., 2003). In that work, the effect of estrogens was tested using relatively large single injections of these hormones without any other stimulus in ovariectomized females. Thus, such injections do not reflect the long-lasting physiological effects of the hormones within the organism. In addition, several studies have shown a positive effect of estrogens on neural proliferation in the SVZ of both prairie voles (Michrotus ochrogaster; Smith et al., 2001) and mice after stroke (Suzuki et al., 2007). In this latter study, estrogens were administered continuously at basal levels, similarly to physiological ones. Also, cerebral ischemia may act as a stimulus to neural proliferation or may induce changes in the direction of cell migration (Goings et al., 2004; Sundholm-Peters et al., 2005; Tonchev et al., 2005). Accordingly, we decided to study possible differences among male and female mice with physiological hormone levels, in the SVZ and also throughout the RMS. Thus, in order for the effects of prolactin to be prevented, our pregnant females were sacrificed on the 14th day of the gestation, when their prolactin levels and their neural proliferation due to this hormone had decreased to the basal range (Shingo et al., 2003). In addition, the prolactin levels of the synchronized females were much lower than those of the pregnant females, in agreement with the findings of Freeman et al. (2000). Accordingly, we suggest that not only prolactin but also estrogens would be the hormones responsible for the increase in neural proliferation in female mice, at least in the RMS.

Differences in neural proliferation between the synchronized females and the other experimental groups were only observed in the SVZ I level. As described above, the pattern of differences among levels did not match the global model only in the group of synchronized females: these females had a higher neural proliferation in the SVZ I level than the other groups. This higher proliferation located specifically in the SVZ I level of the oestrus-synchronized females can be explained in terms of the effect of the progesterone derivatives, DHP and THP, which perhaps only act in pregnant females, diminishing proliferation (Giachino et al., 2004). Progesterone and DHP act through the progesterone receptor, present only in astrocytic cells but not in neuroblasts (Giachino et al., 2004; Melcangi et al., 2001; Melcangi et al., 1999). Nevertheless, THP acts through GABA$_A$ receptors present in neuroblasts and in glial cells (Melcangi et al., 2001). As suggested in previous studies (Giachino et al., 2004), it is possible that the activation of both receptors would be required for proliferation to decrease, and hence only astrocytic proliferating cells, designated B2 and present only in this region (Doetsch et al., 1999; Laywell et al., 2000), would be affected. It is possible that these derivatives might only have been synthesised in the pregnant females, because the other females were sacrificed on the morning after ovulation and hence had not had time to produce them (Giachino et al., 2004). It may therefore be deduced that the negative effect of these derivatives would only act in pregnant females, masking any possible dimorphism.

At SVZ II level, differences were only observed among hormone-injected males and the other groups. This level contains the rostral apex of the SVZ. In this region, neuroblasts change their conformation from clusters to chains of migrating cells (Doetsch et al., 1997; Lois et al., 1996; Peretto et al., 1997). In addition, a higher density of cells was observed at this level, probably due to such a conformational shift. Therefore, it is possible that the increase in cell density could blur other possible differences in the percentages of proliferation.

Regarding serotonergic fibres, differences were found among groups. Our results on serotonergic elements pointed to a higher global fibre density in females than in males, focused in the regions of the RMS. Dimorphic differences in proliferation were also localized in these regions. Moreover, a direct relationship between serotonin and proliferation in the SVZ and the dentate gyrus has been proposed (Banasr et al., 2004), and hence it would be reasonable to speculate that serotonin, as a mediator of the action of estrogen, would activate neural proliferation in the RMS of females. There is compelling evidence in the literature that estrogens act through serotonin as a mediator in the dentate gyrus, increasing neural proliferation (Banasr et al., 2001). In addition, estrogen-induced proliferation in mice with cerebral ischemia is performed by α and β estrogen receptors (Suzuki et al., 2007). However, in the SVZ there are no receptors for estrogens, and hence it is believed that these hormones would act through α and β receptors, although in an indirect way (Suzuki et al., 2007), perhaps mediated by serotonin.

Traditional experiments addressing cell proliferation have focused only on the SVZ, but the RMS also exhibits proliferation, which may be regulated by the same factors as those regulating the SVZ, such as serotonin. The type C cells of the SVZ can act as neural progenitors (Doetsch et al., 1997; García-Verdugo et al., 1998). These cells were originally considered to be present only in this region (Doetsch et al., 1997; García-Verdugo et al., 1998), but they were later discovered in the RMS (Doetsch et al., 2002). In addition, cells originating in the SVZ move not only towards the OB through the RMS, but may also migrate to the accessory olfactory bulb (Bonfanti et al., 1997) or to cortical zones (Goings et al., 2004; Sundholm-Peters et al., 2005). Therefore, the RMS and OB may to a certain extent be independent as regards the regulation of their proliferation rates. We suggest that female sexual hormones might regulate this proliferation in the RMS. In this sense, estrogens would positively regulate proliferation in the RMS through molecules such as serotonin.

We also analyzed programmed cell death in these proliferating regions. The male mice showed more apoptotic cells in the SVZ than the females. Previous authors have reported that estrogens exert a neuroprotective effect, either by themselves or through other substances (Kirschenbaum and Goldman, 1995; Scharfman and Maclusky, 2005; Singh et al., 1995; Wise et al., 2005; Zigova et al., 1998). The SVZ gives rise to cells that proliferate and migrate towards the OB (Altman, 1969; Carleton et al., 2003; Lois and Álvarez-Buylla, 1994). All SVZ cells can be affected by apoptosis: neuroblasts, B cells and C cells. Thus, males could have lower numbers of proliferating cells that reach the RMS. Both the arrival of greater numbers of neuroblasts and the effects of serotonin could explain the higher percentages of proliferating cells in the RMS of female mice.

Conclusions

Differences in neural proliferation exist between male and female mice in the RMS and OB. Our results demonstrate that such differences are possibly due to the action of estrogens through molecules such as serotonin. In addition, males show more apoptotic cells in the SVZ than females, probably due to the action of those hormones. The neuroblasts generated in the SVZ travel through the RMS to the OB to give rise to new interneurons. Such neurons are essential for proper odour discrimination, a sense that (+ in mice?) is essential both in sexual behaviour and in the care of offspring in mice. It is thus reasonable to assume that female physiological hormonal stages such as pregnancy or oestrus would facilitate the contribution of interneurons to the OB, observed as an increase in cell proliferation in the RMS and OB.

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