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Neuroprotective effects of estradiol in hippocampal neurons and glia of middle age mice

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doublecortin;
Ki67

Summary
During aging the hippocampus experiences structural, molecular, and functional alterations. Protection from age-related disorders is provided by several factors, including estrogens. Since aging defects start at middle age, we studied if 17 β-estradiol (E_2) protected the hippocampus at this age period. Middle age (10–12 month old) male C57Bl/6 mice were implanted sc with E_2 (15 μg) or cholesterol pellets. Ten days afterwards they received bromodeoxyuridine (BrdU) 4 and 2 h before killing to study cell proliferation in the dentate gyrus (DG). A pronounced depletion of BrdU+cells in the DG was found in cholesterol-treated middle age mice, accompanied by astrocytosis, and by neuronal loss in the hilus. Middle age mice receiving E_2 showed increased number of BrdU+cells while the other parameters were remarkably attenuated. When steroid treatment was prolonged for 2 months to study migration of cells in the granular layer of the DG, cell migration was unaffected by E_2. However, E_2-treated middle age mice presented higher cell density and increased staining for doublecortin, a marker for differentiating neurons. Thus, from the three basic steps of adult neurogenesis (proliferation, migration, and differentiation), E_2 stimulated progenitor proliferation—even after long exposure to E_2 studied by Ki67 immunocytochemistry—and differentiation towards a neuronal lineage. This result, in conjunction with recovery from other aging indicators as increased deposits of the aging pigment lipofuscin in DG cells, loss of hilar neurons and astrocytosis supports a wide range protection of hippocampal function of middle age mice by estrogenic hormones.

Abbreviations: E_2, 17β estradiol; BrdU, bromodeoxyuridine; DG, dentate gyrus; SGZ, subgranular zone; DCX, doublecortin; GCL, granular cell layer; HPA, hypothalamic–pituitary–adrenal; GFAP, glial fibrillary acidic protein; MA, middle age; chol, cholesterol

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1. Introduction

Aging is accompanied by pathological changes that preferentially target the hippocampus (Smith et al., 2005). Thus, deficits in learning, memory and its associated neurogenesis, changes of neurotransmission, ion channels and electrical activity, altered expression of neuropeptides, growth factors and their receptors, anomalous expression of genes and transcription factors, changes of steroid receptors, vasculopathy, high nitrergic activity and increased oxidative stress, neuronal loss and atrophy, astrogliosis and demyelination account for most hippocampal abnormalities of aging (Ferrini et al., 1999; McEwen, 1999; Driscoll and Sutherland, 2005; Miller and O'Callaghan, 2005).

A typical feature of aging is the reduction of hippocampal neurogenesis (Cameron and McKay, 1999; Kuhn et al., 1996; Kempermann et al., 2002; Heine et al., 2004). In the adult, this process is restricted to the subventricular zone and the subgranular zone (SGZ) of the dentate gyrus (DG). Progenitors in the DG proliferate, migrate into the granular cell layer (GCL) and differentiate into mature granule cells (Cameron and McKay, 1999; Cameron et al., 1993; Heine et al., 2004). Functionally, neurogenesis is associated with learning and memory and acquisition of a fear-conditioned response (Gould et al., 2000; Shors et al., 2002). Since newly-formed neurons may optimize interconnections between the DG and the CA3 pyramidal subfield, the whole hippocampal function may be influenced by changes of neurogenesis (Kempermann et al., 2002).

Steroid hormones strongly influence neurogenesis besides other hippocampal parameters. Estrogens are neuroprotective hormones (Behl, 2002; García-Segura et al., 2001; McEwen et al., 2001) and they positively control neurogenesis. For instance, uptake of the thymidine analog bromodeoxyuridine (BrdU) by proliferating cells of the DG is higher in proestrus than in estrus rats, suggesting the participation of endogenous hormones, whereas BrdU+cells are more abundant in ovariectomized-estradiol (E_2) replaced rats than in ovariectomized-vehicle treated rats (Tanapat et al., 1999). The increase in proliferation is transient, and diminishes in animals subjected to prolonged ovariectomy or chronically overloaded with estrogen (Ormerod et al., 2003). A gender difference has been suggested, because in males the response of hippocampal neurogenesis to E_2 is attenuated (Galea et al., 2006). In our experience, a ceiling effect probably accounts for the failure of E_2 to increase granule cell proliferation in normal male mice. In contrast, the response to E_2 is noticeable in diabetic male mice, which show extremely low cell proliferation rates under basal conditions (Saravia et al., 2004, 2006; Beauquis et al., 2006).

It is well accepted that in aging animals, proliferation and migration of newborn cells in the DG is strongly reduced (Heine et al., 2004). In part, this reduction may be exacerbated by a dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis (van Eekelen et al., 1991; Ferrini et al., 1999; Sapolsky, 1999; Dalm et al., 2005). Hyperfunction of the HPA axis increases the secretion of adrenal steroids, which negatively impact on hippocampal integrity and on cell proliferation and differentiation in the DG (Miller and O'Callaghan, 2005; Wong and Herbert, 2005). Progenitors in the DG express glucocorticoid and mineralocorticoid receptors (Wong and Herbert, 2005). Therefore, hippocampal neurogenesis and HPA axis activity are inversely correlated, suggesting that the low hippocampal neurogenesis of senescent animals may be an index of glucocorticoid-mediated neurotoxicity (Cameron and McKay, 1999; Kempermann et al., 2002).

That estrogens positively control neurogenesis has been mostly informed for young animals (Tanapat et al., 1999, 2005; Ormerod et al., 2003), while few studies evaluated a similar role in old animals. In one study (Perez-Martin et al., 2005) 22-month-old ovariectomized rats received prolonged treatment with estradiol valerianate or phytoestrogens from soy bean extract. The authors concluded that cell proliferation in the old brain remains responsive to natural estrogens and phytoestrogens. Although in an indirect manner, a second report demonstrated that infusion of insulin growth factor type I (IGF-1) to 22-month-old rats increases both neurogenesis and blood levels of E_2, suggesting steroid participation in the cell proliferation of this age group (Darnaudery et al., 2006). Interestingly, hippocampal neurogenesis starts to decline well before old age. For instance, cell proliferation and migration through the GCL is high in 2-week-old rats, it weakens at 1.5 month of age, and is drastically inhibited in 12 month (middle age) and 24 month (old age) rats (Heine et al., 2004). Considering this early decline, studies to elucidate estrogenic effects on the steps leading to neuronal maturation in the DG and on other hippocampal indicators of aging remain an important subject. This issue has clinical relevance, because the Women Health Initiative (WHI) randomized trial claimed that estrogen alone increases the risk of developing mild cognitive impairment (Resnick et al., 2006), a process considered hippocampal-dependent. The WHI trial has been criticized on the grounds that it recruited old postmenopausal women (65–79 years old), an age span when estrogen responsiveness diminishes (Foster, 2005). Clinical trials support the effectiveness of hormone-replacement therapy in prevention rather than improvement of mental deterioration (Henderson et al., 1994; Wang et al., 2000). Since middle age animals are fully responsive to E_2 (Brewer et al., 2006; Wise, 2006), this age period seems appropriate to counteract the development of age-associated neuropathology. Thus, middle age provides an attractive window of time to explore potential modulation of changes associated with aging, and paradoxically, the literature is not abundant in this period of life.

To fully appreciate hormonal effects on the middle age hippocampus, it seems important to expand the study to other age-sensitive parameters besides neurogenesis. A typical biomarker of the aging brain is the astrocyte hypertrophy, with increased expression of the glial fibrillary acidic protein (GFAP) (Goss et al., 1991; Nichols et al., 1993; David et al., 1994). Estrogens produce a down-regulatory effect on the astrocytosis with high GFAP expression of the brain of very old rats (22–26 months at the time of killing) and young rats receiving castration, traumatic or excitotoxic lesions (Day et al.,1993; Garcia-Ovejero et al., 2002; Lei et al., 2003). Another hallmark of aging is lipofuscin, an autofluorescent pigment that accumulates inside neurons due to increased oxidative stress (Keller et al., 2004). E_2 treatment decreases brain lipofuscin in 12-, 18- and 24-month-old rats, according to one study that included

several brain regions but not the hippocampus (Moorthy et al., 2005). A third aging marker is the neuronal population of cells of the hilar region, which suffer a pronounced loss in old animals (Cadacio et al., 2003). Hilar neurons are extremely vulnerable to excitotoxic or ischemic hippocampal injury (Azcoitia et al., 1998). It is unknown if estrogens prevent the hilar neuronal loss during aging, although a rescue effect of acute or chronic E_2 administration follows toxin-induced degeneration of neurons in this region (Azcoitia et al., 1998; Picazo et al., 2003).

In the present investigation, the effects of estrogen on the hippocampus of middle age mice was analyzed at several levels. We first demonstrated that E_2 treatment stimulated progenitor proliferation, according to BrdU incorporation, and differentiation towards a neuronal lineage, according to doublecortin (DCX) immunostaining. As an extension of these findings, we also demonstrated that E_2 treatment of middle age mice decreased the number of GFAP astrocytes, restored hilar cell number and diminished lipofuscin deposits, suggesting a wide range estrogen neuroprotection in the hippocampus of middle age animals.

2. Methods

Male C57Bl/6 mice from the Instituto de Biologia y Medicina Experimental colony were housed under conditions of controlled humidity and temperature (22 °C), with lights on from 07:00 am to 07.00 pm. Animals were divided into a 4-month-old young group and a 10–12 middle age group (MA). Two experiments (Exp.) were designed, employing different times and doses of hormone treatment to explore the effect of estrogen treatment on the hippocampus. Exp. I employed a short course of E_2 treatment, in which case a single pellet containing 15 μg of E_2 diluted to 200 μg with cholesterol (chol) (Sigma, St. Louis, MO) or chol only was inserted sc into MA mice and left in place for 10 days. Mice killed at this time point were used to determine cell proliferation by counting BrdU+cells, the number of GFAP+hippocampal astrocytes and hilar neurons in the DG. Exp. II was designed to study the effects of long E_2 treatment, in which case MA mice received sc a single pellet containing 150 μg E_2 or chol for 60 days. Mice killed at this time point were used to study cell proliferation using Ki67 immunocytochemistry, and also progenitor survival and migration into the granular cell layer (GCL) by counting BrdU+cells labeled 21 days before sacrifice. In Exp. II we also used immunostaining for the neuroblast marker DCX, and determined the number of hippocampal GFAP+astrocytes, hilar neurons and cells containing lipofuscin deposits. Animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals (Assurance Certificate ♯A5072-01) and protocols received the approval of the Institutional Animal Care and Use Committee.

In order to test the effectiveness of E_2 treatment, some endocrine parameters were evaluated. Young and MA animals were weighted at the end of the experiment but no significant differences were found between experimental groups. As expected, E_2 treatment during 2 months produced testicular atrophy: MA$\pm E_2$: 0.170 ± 0.007 g vs. MA+chol: 0.203 ± 0.003 g ($p<0.05$) and pituitary gland hypertrophy: MA$\pm E_2$: 3.94 ± 0.108 mg vs. MA+chol: 2.44 ± 0.136 mg ($p<0.01$). Serum estradiol was measured at the end of the experiment using a commercial kit (Coat-a-Count TKE21, Diagnostic Products Corp., Los Angeles, USA). Results were calculated after logit–log transformation of displacement curves, and showed a 36-fold increase of serum E_2 in MA estrogenized males: 550.83 ± 77.94 pg/ml respect of steroid naïve MA animals: 15.12 ± 2.34 ($p<0.001$). Serum corticosterone, measured by a competitive protein binding technique (Ferrini et al., 1999) was significantly decreased by E_2 treatment of MA mice (6.9 ± 3.6 ng/ml) with respect to steroid-free MA animals (27.6 ± 8.7, $n = 6$ mice per group, $p<0.001$).

To carry out immunohistochemical determinations, mice were deeply anesthetized with ketamine (33.3 mg/100 g body weight) and transcardially perfused with 30 ml of 0.9% NaCl solution followed by 50 ml 3% PFA in phosphate buffer (PB), pH 7.4. Brains were incubated overnight in 3% PFA. On the next day, they were transferred to Tris-buffered 0.9% NaCl (TBS), pH 7.4, and 60 μm coronal sections were prepared using a vibrating microtome and stored in cryoprotectant solution made of ethylene glycol–glycerol–0.1 M sodium phosphate buffer pH 7.4 (0.6:0.5:1.0) at −20 °C until used for immunocytochemistry and morphological analysis. Consistency of the procedures was intended by staining sections from all experimental groups simultaneously. This method eliminated conflicts that could arise using different batches. To minimize interexperimental variability, conditions were kept rigorously identical throughout the assays. The investigators who processed the cell counting were blinded to the procedure.

2.1. BrdU administration and immunocytochemistry

Exp. I: To study cell proliferation in the DG, mice received i.p. injections of 5-bromo-2′-deoxyuridine (BrdU) (Sigma, 130 mg/kg BW dissolved in 0.9%saline) 4 and 2 h before sacrifice (outlined in Fig. 1). We followed previously published procedures for detection of BrdU+cells (Saravia et al., 2004, 2006; Beauquis et al., 2006).

Briefly, coronal sections were rinsed, incubated in prewarmed 50% formamide/2 × sodium citrate/sodium chloride at 65 °C for 10 min, rinsed in 2 × sodium citrate/sodium chloride for 10 min, incubated in 2 N HCl at 37 °C for 30 min, rinsed in 0.1 M boric acid, pH 8.5, for 10 min, rinsed and blocked for 30 min in TBS with 0.1% Triton X-100 and 10% goat serum. Sections were then incubated for 48 h with monoclonal anti-BrdU antibody made in rat (1:200, Accurate Chemicals, Westbury, NY) biotinylated anti-rat IgG antibody (1:200, Sigma), processed according to the ABC kit instructions (Vector Labs, CA, USA) and developed with 3,3′-diaminobenzidine (DAB) 0.5 mg/ml, 0.05% H_2O_2. Sections were counterstaining with cresyl violet, dehydrated, cleared and mounted with Permount (Fisher Chemical, USA). Nonspecific staining was assessed in the absence of primary antibody.

Exp. II: In this case, mice received a single BrdU injection (250 mg/kg BW) 21 days before sacrifice, as explained in Fig. 1 and BrdU immunodetection followed the above-mentioned steps.

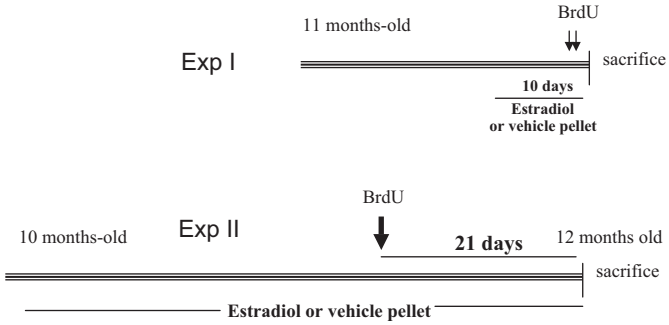


Figure 1 Experimental design. In Exp. I, 11-month-old middle age (MA) mice received for 10 days a single 15 μg E_2 pellet (sc) diluted with cholesterol (chol) or chol only. Two and 4 h before killing BrdU (130 mg/kg, arrows) was injected to determine cell proliferation. Exp. I was also used to study GFAP+astrocytes and hilar neurons in the DG. In Exp. II, 10 month-old mice received for 60 days 150 μg E_2 or chol pellets (one pellet was placed at day 1 and other identical pellet was placed at day 30). Exp. II was designed to study cell proliferation (Ki67 immunocytochemistry), for progenitor survival and migration into the granular cell layer (GCL) by labeling with BrdU (a single injection of 250 mg/kg) 21 days before killing, to label neuroblasts with doublecortin (DCX) and to analyze effects on GFAP+astrocytes, hilar neurons and lipofuscin deposition.

2.2. Quantification of BrdU-immunoreactive cells

In Exp. I and II, BrdU-positive cells were counted on every 8th section throughout the entire rostrocaudal extension of both halves of the DG (SGZ/GCL), corresponding to Plates 6–23, A1100–A3450 from the stereotaxic atlas of the mouse brain (Lehmann, 1974). The same area and number of sections ($n = 6$ per animal) were studied from each experimental group. All BrdU+cells were counted with a 40 × objective in an Olympus BH-2 microscope. Cell counts were restricted to the GCL and the SGZ. The SGZ was defined as a two-nucleus-wide band below the apparent border between GCL and the hilus. The total number of BrdU-labeled cells was estimated according to the optical dissector method (West, 1993; Coggeshall and Lekan, 1996; Howard and Reed, 1998): positive nuclei located in the uppermost focal plane were ignored, whereas immunopositive nuclei completely filled with DAB product or showing typical patches of variable intensity were counted. Therefore, positive immunoreactive nuclei that came into focus and focusing down through the thickness of the section were considered for the study. The number of BrdU-immunoreactive nuclei was multiplied by a factor of 8 to estimate the total number of BrdU-labeled cells in each specified area.

In Exp. II, BrdU+cells were also counted in the SGZ, the hilus and three divisions of the GCL: inner (1/3), close to the SGZ, middle (2/3) and outer (3/3) limiting with the molecular layer. This method, which followed the migratory pathway from the SGZ through the GCL, represented the number of BrdU+surviving cells in the migratory route (Brown et al., 2003; Cameron and McKay, 1999).

2.3. Doublecortin immunocytochemistry

A doublecortin (DCX) antibody was employed to label differentiating cells (neuroblasts) (He et al., 2005). To this end, coronal brain sections were first exposed to methanol:H_2O_2 (100:1) during 10 min at RT, washed and blocked for 30 min in phosphate buffer saline (PBS) containing 10% rabbit serum at 37 °C. Sections were incubated with a goat polyclonal DCX antibody (1:250, sc-8066, Santa Cruz Labs.) followed by a biotinylated anti-goat IgG made in rabbit (1:200, Sigma) and processed following the ABC kit instructions. For development, we used DAB at 0.25 mg/ml, 0.05% H_2O_2 at RT. Sections were finally mounted, dried, dehydrated and mounted with Permount (Fisher Chemical, USA). Nonspecific staining was assessed in the absence of primary antibody. DCX-positive cells were counted using stereological methods (Coggeshall and Lekan, 1996; Howard and Reed, 1998; West, 1993) as specified above for BrdU+cells. Six sections per mice corresponding to 6 animals were studied for each experimental group. All positive cells were counted with a 40 × objective in an Olympus BH-2 microscope. Cell counts were restricted to the GCL and the SGZ of the DG. The number of DCX-immunoreactive cells counted in the GCL/SGZ was multiplied by 8 to estimate the total number of DCX-labeled cells in the dentate gyrus. Additionally, length of DCX immunopositive processes was measured using the Optimas Bioscan software for images. The program overdrew the soma-associated cell processes giving the corresponding length. Six soma-associated processes were counted per slice containing the dorsal DG. From 8 slices per animal and 7 mice per group we obtained the average length per animal and per experimental group. This method was used for counting processes of MA mice with or without E_2 treatment. In contrast, only the number of DCX+cells was calculated for young animals, because a marked overlapping of immunostained processes prevented determination of length in this age group.

2.4. Ki67 immunocytochemistry

For Ki67 immunoreaction, a slight modification of a published method was followed (Heine et al., 2004). Sections were washed three times in TBS, pH 7.4 in a shaker, incubated in 0.01 M citrate buffer pH 6.0 at 90 °C during 40 min, left to cool at RT, and further incubated with methanol:H_2O_2 (100:1) in TBS during 15 min at RT. After washes in TBS, sections were blocked for 30 min in TBS containing 2% nonfat milk at RT and incubated overnight at

4 °C with a polyclonal anti Ki67 antibody made in rabbit (1:2000, Novocastra) diluted in 0.5% Triton X-100 TBS 1% goat serum. After 3 washes in TBS, sections were incubated with a biotinylated anti-rabbit IgG made in goat (1:200, Sigma) in 0.5% Triton X-100-PBS, 1% goat serum for 90 min in a shaker at RT. After 3 washes in TBS, they were processed following the ABC kit and developed using 0.5 mg/ml DAB, 2.5% nickel, 0.05% H_2O_2 at RT. Sections were dehydrated and mounted as specified before. Nonspecific staining was assessed in the absence of primary antibody. Counting of Ki67+cells was identical to that described of DCX+cells.

2.5. GFAP immunocytochemistry

The number of GFAP immunopositive cells was assessed in the hilus of the DG, following previously published procedures (Saravia et al., 2006). Free-floating sections were exposed to a 1/300 dilution of rabbit anti-GFAP polyclonal antibody (G-9269, Sigma), and developed using a biotinylated goat anti-rabbit complex (Vectastain ABC Elite Kit), DAB was used for development at 0.5 mg/ml in 0.05% H_2O_2. Data were obtained for each section from 6 to 8 sections per animal, using 6–7 animals per group. Computerized image analysis was used for counting GFAP immunoreactive cells. Cell densities (number of cells/unit area $= 80 \times 10^3\,\mu m^2$) were determined in 6–9 anatomically matched areas. Data were expressed as the mean number of labeled cells $\pm$ SEM.

2.6. Morphometric analysis of hilar cell number

The number of cresyl violet stained neurons in the hilus of the DG was estimated by the optical dissector method (Howard and Reed, 1998). Stained nucleated cells that came into focus and focusing down through the dissector height were counted and the total number of cells in the hilus was informed, the volume corresponding to the dissector used was $150 \times 10^3\,\mu m^3$, 6 sections from each mouse were used, 350 μm being the distance between them.

2.7. Measurement of granular cell layer reference volume and hilar volume

Volumes of the GCL and the hilar region of the DG were estimated separately in 50 μm sections stained with cresyl violet and one every 8 sections were selected for the analysis. Computer-assisted image analysis was employed on the basis of the Cavalieri principle (Gundersen and Jensen, 1987). Structures were outlined, and the computed areas were summed and multiplied with the intersection distance and with the thickness of the sections. Volumes were expressed per mm^3 corresponding to an average of both halves of the structure (Magariños and McEwen, 2000).

2.8. Lipofuscin autofluorescent deposits

Fifty micrometer coronal sections obtained with the vibratome were mounted on slides using PBS:glycerol (50:50) and observed directly under fluorescence microscopy. Lipofuscin autofluorescent cells were selected according to Kempermann et al. (2002), in that only cells in which the deposits obscured more than half of the nucleus were counted. Pigment loaded cells were counted on 12 hemisections of the DG per animal and results were expressed as the mean ($\pm$SEM) number of lipofuscin positive cells per GCL.

2.9. Statistical analysis

For all studies, statistical analysis was performed using a one way ANOVA, followed by Bonferroni's post hoc test, with $p < 0.05$ as the criterion for statistical significance.

3. Results

An outline of the protocols followed in Exp. I and Exp. II is given in Fig. 1.

3.1. Cell proliferation after short E_2-treatment (10 days) in middle age mice

In order to study cell proliferation in the DG, MA mice implanted with chol or E_2 pellets during 10 days received BrdU injections 4 and 2 h before sacrifice (Fig. 1). Figure 2 shows morphological evidence for the pronounced depletion of BrdU+cells found in the DG (SGZ/GCL) of MA mice (Fig. 2A). In contrast, scattered BrdU+cells were clearly visualized (arrows) in the SGZ of both upper and lower blades of the DG of E_2-treated MA mice (Fig. 2B). We demonstrated that about 250 cells incorporated the thymidine analog in the hippocampus of steroid-naïve MA mice, whereas the number of BrdU+cells doubled after E_2 treatment ($p < 0.02$ vs. untreated group). Figure 2C illustrates a cluster of BrdU+cells in the SGZ/GCL of E_2-treated MA mice, suggesting active mitoses; such clusters were never seen in chol-implanted MA mice. Notwithstanding the significant stimulation of cell proliferation in E_2-treated MA animals, rates for this group were below the $\approx$1100 BrdU+cells per DG reported previously for young mice (Saravia et al., 2004, 2006; Beauquis et al., 2006). These data were included for comparison in Fig. 2.

3.2. Cell survival and migration after E_2-treatment prolonged for 2 months (Exp. II Fig. 1)

Exp. II introduced two variables: (a) E_2 treatment was increased to 150 μg and prolonged for 2 months, (b) dose of BrdU was raised to 250 mg/kg and given 21 days before killing. BrdU labeling was determined in SGZ/GCL (shown in Fig. 3A) and in cells in a migratory pathway from the SGZ to the outer third of the GCL. Data were expressed as % BrdU+cells per zone/total number of BrdU labeled cells. Whereas most BrdU+cells remained in the SGZ, small quantities distributed along the inner (1/3), middle (2/3) and outer (3/3) zones of the GCL and in the hilus of the DG (Fig. 3B). The mentioned subfields corresponding to the GCL are illustrated in the microphotography included in Fig. 3C. However, no significant differences were obtained between chol-implanted or E_2-implanted MA mice at any migratory distance considered. A trend existed for the total number of BrdU+cells being higher in E_2-treated MA mice than in

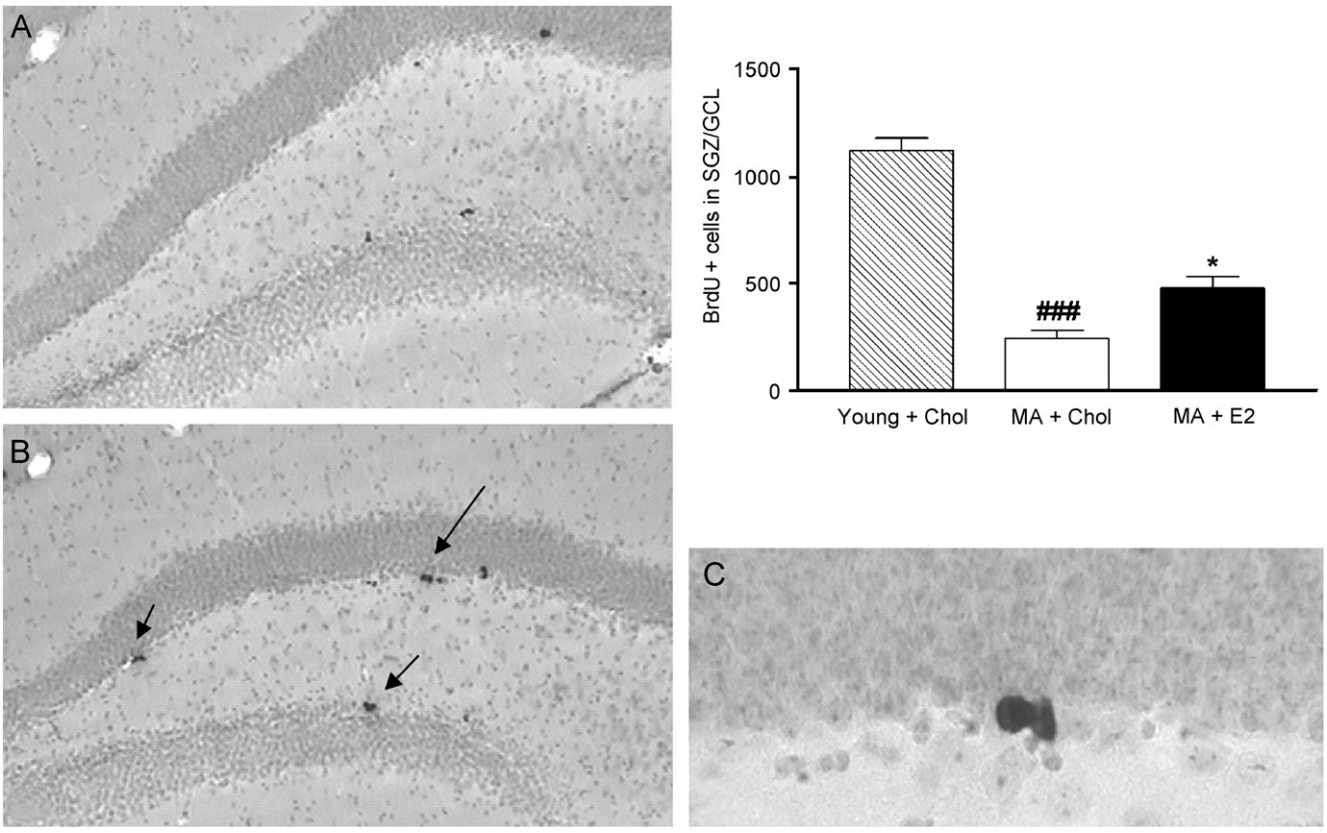


Figure 2 Data for cell proliferation in Exp. I. (A) and (B) Representative photomicrographs of the SGZ/GCL of middle age mice receiving chol (A) or short E_2 treatment (B). BrdU+cells were scarce in a chol-treated MA mouse but clearly visible in an E_2-treated MA mouse (arrows in B). (C) A mitotic figure in an E_2-treated MA mouse. Quantitative analysis demonstrated that after E_2 treatment (dark column) the number of BrdU+cells was significantly higher than in chol-treated MA mice (white column; $^*p<0.05$). Data obtained for young mice are shown for comparison (stippled column) $\#\#\#p<0.0001$ vs. Young+chol. Magnification 40 $\times$ for (A)–(B), and 400 $\times$ for (C).

steroid-free mice, but differences were not significant. Therefore, neither the migratory pattern nor the number of BrdU+cells surviving 21 days after BrdU administration were modified after prolonged E_2 treatment of MA mice.

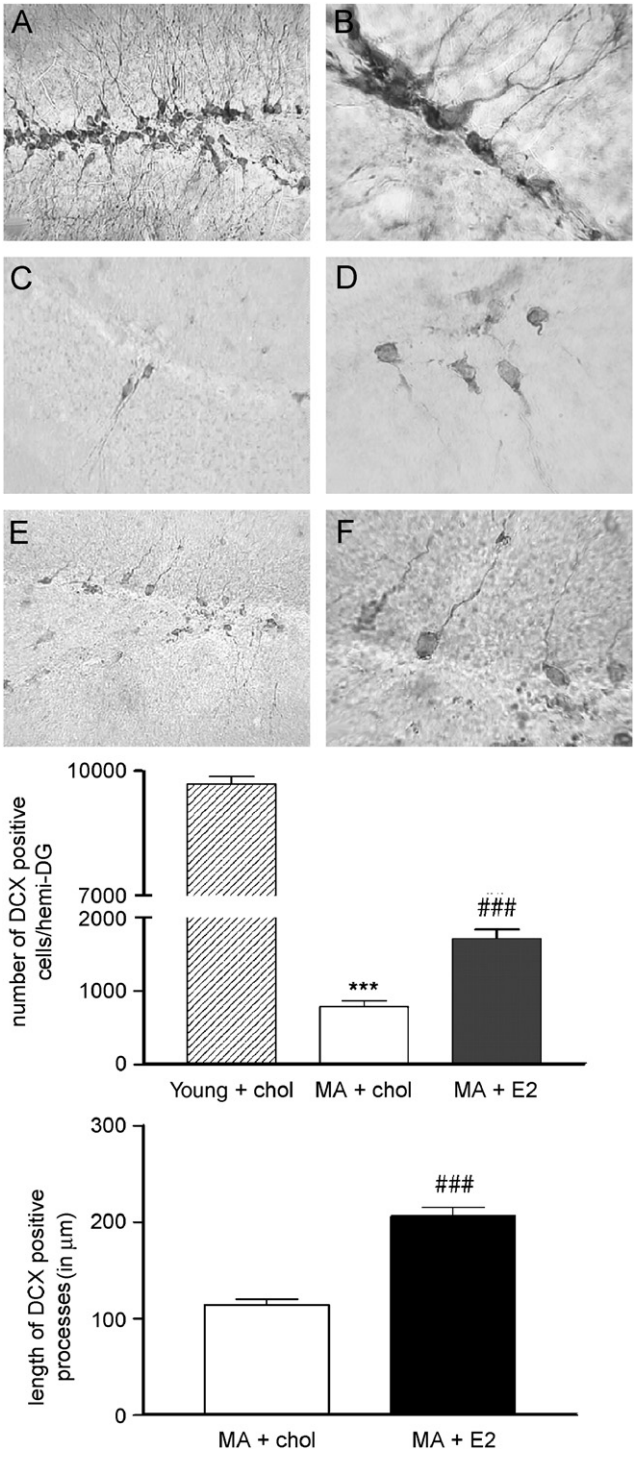
3.3. Cell differentiation in E_2-treated MA mice

Staining for the neuroblast marker DCX was carried out in sets of sections obtained from animals in the cell migration study. For this experiment, a group of young 4-month-old mice was included, because we had no previous data on this age group using the DCX antibody. Low power microscopy of young animals demonstrated numerous aggregates of highly branched DCX+cells located in the SGZ and the GCL (Fig. 4A), few labeled cells in MA mice but increased number of DCX-stained cells in the MA group receiving E_2 (Fig. 4C and E, respectively). In a high power view (Fig. 4B), the staining profile of DCX+cells of young mice suggest their differentiation towards a neuronal phenotype, i.e., cells showed a round to triangular shape and long stained processes. DCX+cells in MA mice, in contrast, looked pale and atrophic, with a hardly visualized or short stained process (Fig. 4D). After E_2 treatment, DCX+cells of MA mice presented a round perykarium and a clearly visible neuritic process (Fig. 4F). These qualitative observations were done using stereological methods and computerized image analysis for quantitation of the number of DCX+cells and the length of their processes, respectively (Fig. 4 bottom). Thus, a 10-fold reduction of DCX+cells was determined in untreated MA mice ($p<0.0001$ vs. young mice), whereas a doubling of DCX+cells followed E_2 treatment ($p<0.0001$ vs. untreated MA). DCX+cells in E_2-treated MA mice, however, remained significantly lower than the young group. Concerning the DCX+cell processes, steroid treatment during 2 months significantly enhanced the length of neurites in MA mice ($p<0.0001$). The morphological appearance of the DCX+processes in the MA mice exposed to E_2 was closer to the young group, while those corresponding to untreated MA mice were shorter and convoluted. Thus, data from Exp. II confirmed that E_2 treatment significantly stimulated cell differentiation, although the morphology and number of DCX+cells of the hormone-stimulated MA group was behind the younger counterpart.

3.4. Effect of long E_2 treatment on DG proliferation in middle age mice

Exp. II also explored if DG proliferation remained hormone sensitive after E_2 exposure for 2 months. As the animals were injected with BrdU, 21 days before sacrifice, we

by measuring the number of autofluorescent cells per hippocampal GCL. This abnormal pigment, accumulated in the cytoplasm of neurons of untreated MA mice (Fig. 8, top), was much less noticeable in the steroid-treated mice, while in young animals was not frequently found. Thus, the number of autofluorescent cells per GCL was significantly reduced in the GCL of E_2-treated MA mice with respect to steroid-naïve mice ($p = 0.03$), as it can be observed in the lower graph of Fig. 8.



4. Discussion

Middle age represents a special period of life useful to detect events in the route to senescence. In this work, we explored the effectiveness of estrogen treatment at MA to prevent or attenuate the hippocampal abnormalities associated with physiological aging. To this end, neuronal parameters were approached, including the different steps of neurogenesis, the number of hilar neurons and lipofuscin loading, and one glial cell parameter—GFAP expression by astrocytes—which are reportedly disturbed in senescent animals. The results indicated that out of the three steps of adult neurogenesis (proliferation, migration, and differentiation), E_2 stimulated progenitor proliferation and differentiation. The effect on neurogenesis was accompanied by decreased astrocytosis, a reduced deposition of lipofuscin and an increased number of hilar neurons in the DG and GCL.

In the first place, we showed that a moderate dose of E_2 given for 10 days doubled cell proliferation in DG, suggesting that this neurogenic zone remains hormone sensitive through MA. However, estrogen stimulated level of BrdU incorporation at MA was still lower than that obtained for young male animals, which average $\approx$1100 BrdU+cells per DG using identical labeling and counting protocols (Saravia et al., 2004, 2006; Beauquis et al., 2006). Therefore, other factors besides steroids may be needed for full recovery of cell proliferation, or recovery of plasticity may be limited in MA mice. In contrast to the stimulatory effect on cell proliferation, E_2 did not change migration into the GCL. This result was not unexpected, because previous reports suggested that estrogen effects can be transient and do not go beyond the proliferation step (Tanapat et al., 1999; Ormerod et al., 2003). In this respect, MA animals behaved like young animals studied by other investigators.

It is possible that the E_2 effects on cell proliferation of MA mice may be of functional significance, because when steroid treatment was prolonged to 2 months, differentiation of cells towards a neuronal lineage was highly stimulated. In our investigation, progenitors were labeled for DCX, a microtubule binding protein transiently expressed by proliferating cells and newly generated neuroblasts (Brown et al., 2003). As demonstrated by us here and also by others (Brown et al., 2003; McDonald and Wojtowicz, 2005), the incidence of DCX+cells was markedly decreased in aging rats and MA mice. However, E_2 treatment not only

Figure 4 Morphology and quantitative analysis of DCX+cells (differentiating progenitors) in the DG of young mice and middle age mice with and without E_2 treatment (Exp. II). Numerous DCX+cells with abundant stained processes characterized the young group, as observed in low (A) and high power (B) views, in sharp contrast with the atrophic appearance of untreated MA mice (C), (D). In the E_2-treated MA group, DCX+cells were more abundant with staining of cell processes (E), (F). Magnification: (A), (C), (E) 400 $\times$; (B), (D), (F) 1000 $\times$ Upon quantitation, DCX cell number in MA control mice was significantly lower than young group (***$p <$ 0.0001) while length of processes was significantly reduced in MA mice (white column) respect of E_2-treated MA mice (dark column)(###$p < 0.0001$ for cell number and for processes vs. MA+chol). Still, DCX+cell number of E2-treated MA mice did not reach the level of young mice.

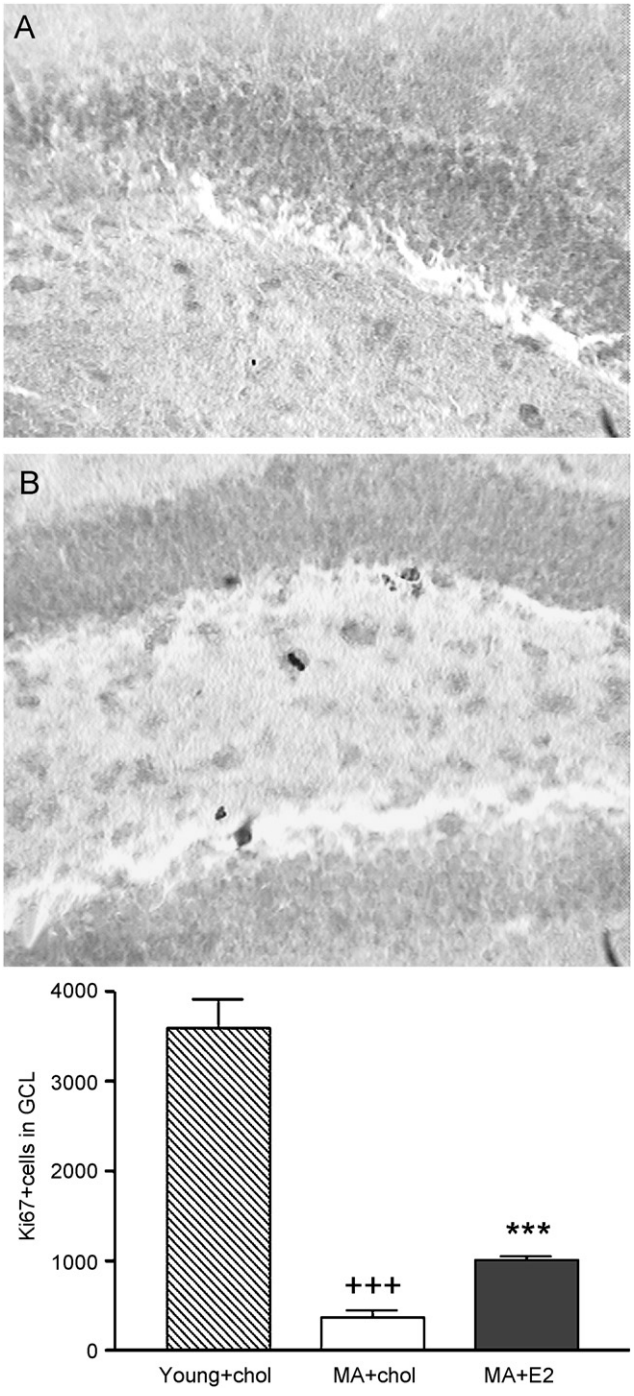


Figure 5 Cell proliferation in DG using the Ki67 antibody (Exp. II). (A) and (B) constitute representative photomicrographs of Ki67+cells in a MA mouse (A) and an E_2-treated MA mouse (B) Magnification: 400 $\times$. Quantitation demonstrated a significant reduction of Ki67+cells in both MA groups respect of young animals (stippled column; $+++p<0.001$ vs. Young+chol); however the E_2-treated MA mice (dark column) showed significantly more immunopositive cells than chol-treated MA animals (white column, $***p<0.0001$ vs. MA+chol).

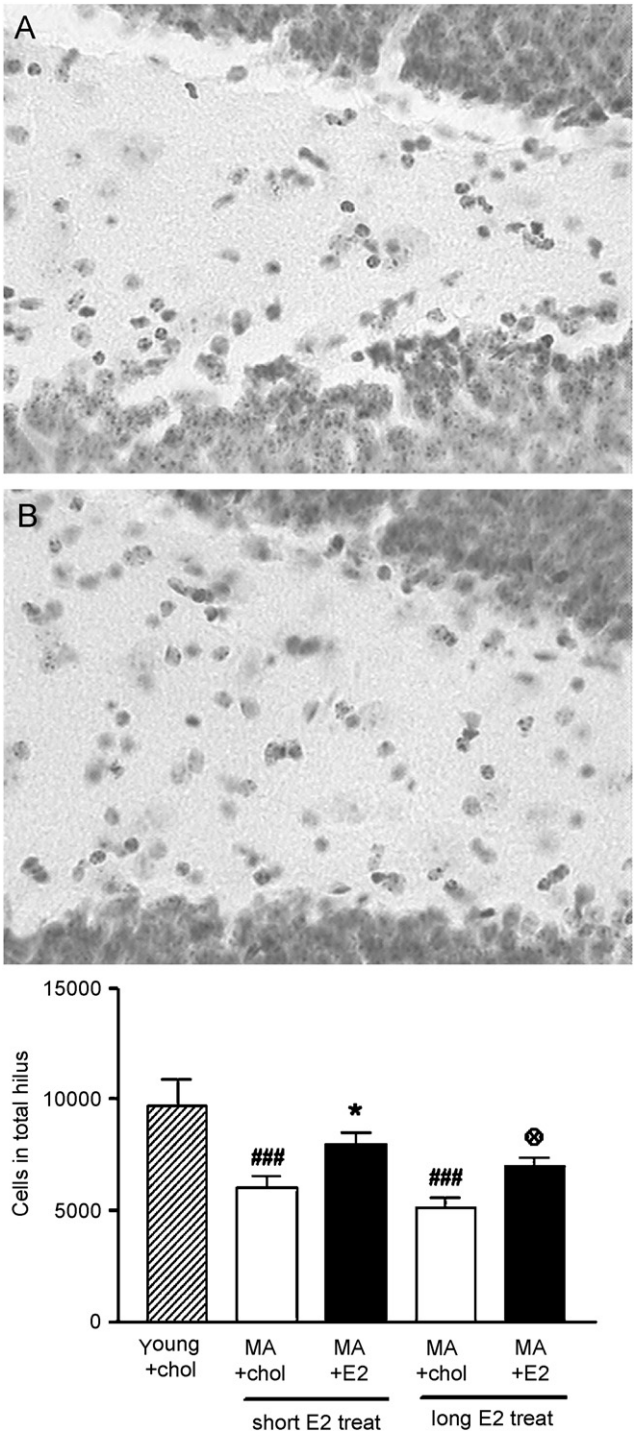


Figure 6 Hilar neuronal number for Exp. I and Exp. II. (A) and (B) represent cresyl violet stained sections (magnification 400 $\times$) from a MA mouse (A) and a MA mouse subjected to prolonged E_2 treatment (B). The lower graph shows that both the short (Exp. I) and long (Exp. II) E_2 treatment (dark columns) produced higher hilar cell number compared to their respective untreated MA group (white columns). The number of hilar cells counted in young mice is showed for comparison (stippled column) $###p<0.0001$ vs. Young+chol; *and $\otimes$ $p<0.05$ vs. MA+chol short and long E_2 treatment, respectively.

increased the number of DCX labeled cells, but also increased staining of the perikaryon and neurite processes oriented parallel and into the GCL. From literature reports,

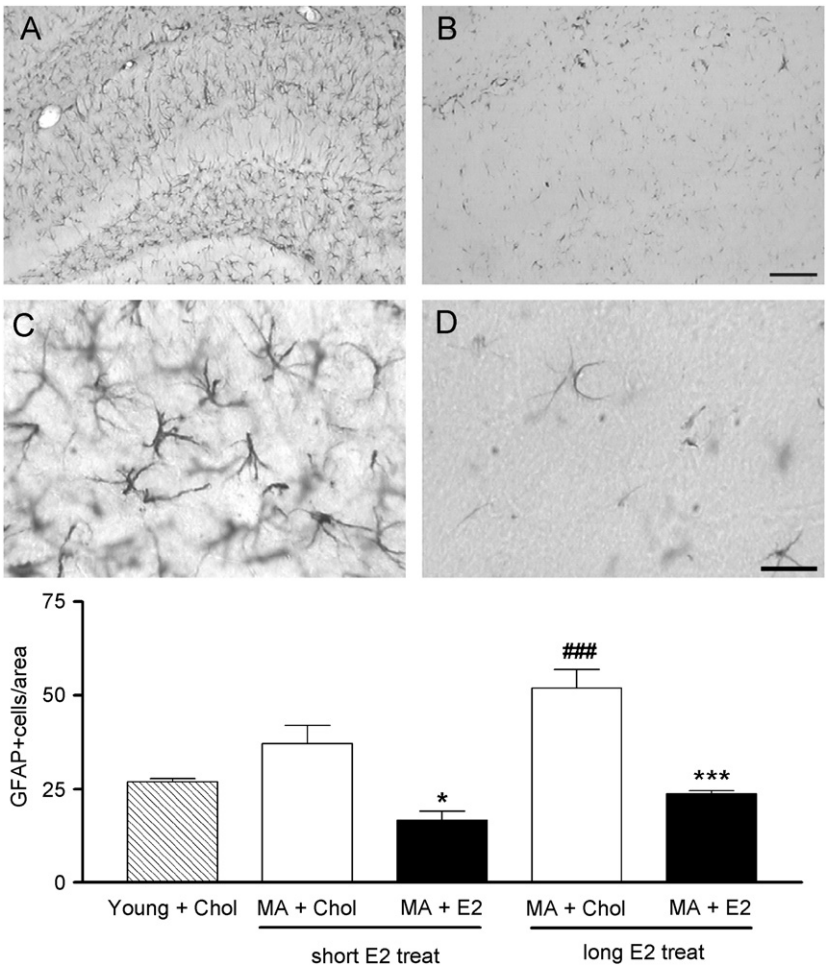


Figure 7 Photomicrographs showing glial fibrillary acidic protein-positive (GFAP) cells in dentate gyrus from a MA mouse in low ((A), 100 ×) and high power ((C), 600 ×) views and corresponding images from a MA mouse receiving prolonged E_2 treatment (B), (D). Note the marked GFAP-immunopositive astroglial reaction in (A)–(C) and its attenuation in (B)–(D). Cell densities (number of cells/unit area $= 80 \times 10^3\ \mu m^2$) were determined in 6–9 anatomically matched areas. The bottom graph depicts quantitative analysis of GFAP-positive cells per area in Young mice+chol (stippled column) MA+chol (white columns) and MA+E_2 treated mice (dark columns) under both E_2-treatment regimens. Statistical analysis demonstrated for MA+E_2 vs. MA+chol groups $^*p<0.05$ and $^{***}p<0.0001$ for short and long hormone treatment, respectively, and ### MA+chol during 2 months vs. Young+chol $p<0.0001$.

it is known that immature granule cells rapidly extend axons into CA3 and become integrated into the hippocampal circuit, where they could influence hippocampal function at early stages of neurogenesis (Hastings and Gould, 1999; Kempermann et al., 2002). Therefore, after E_2 stimulation of cell differentiation, some missing functions may resume in the hippocampus of MA mice, since the enlarged DCX+processes found in steroid-treated MA mice could reinforce the synaptic interconnections of the hippocampal circuit. In this case, changes observed in the length of cell processes after E_2 treatment seems to be independent of the dose of steroid used and the duration of the exposure.

Furthermore, the stimulation of cell proliferation in the DG of MA mice may be viewed as another example of E_2 neuroprotection. Mechanistically, E_2 effects on the hippocampus might be partly mediated by classical estrogen receptors (ER). Thus, the presence of mRNAs for the isoforms ERα and ERβ colocalize with markers of cell proliferation and differentiation (Shughrue and Merchenthaler, 2000; McEwen et al., 2001). The blockade of neurogenesis by administration of an ER antagonist (Perez-Martin et al., 2005), further indicate a role for the intracellular receptor, although pathways independent of the classical ER have been considered to be part of this event (McEwen et al., 2001; Behl, 2002). Estrogen also regulates the expression of IGF-I and of brain-derived neurotrophic factor (BDNF), activate cellular cascades involving IGF-I receptors and BDNF receptors (Cardona-Gomez et al., 2001; Perez-Martin et al., 2005; Scharfman and Maclusky, 2005). These growth factors promote proliferation and neuronal differentiation (Anderson et al., 2002), and their interaction with E_2 may explain in part the stimulatory effects on neurogenesis. This interaction does not exclude that effects on neurogenesis comprises additional activities of E_2 in the brain. In this sense, E_2 neuroprotection involves multiple mechanisms, such as prevention of excitotoxicity and inflammation, antioxidant effects, inhibition of apoptosis and stimulation of the anti-apoptotic gene Bcl_2, regulation of cholinergic neurotransmission and dendritic remodeling, among others (Diaz-Brinton et al., 2000; García-Segura

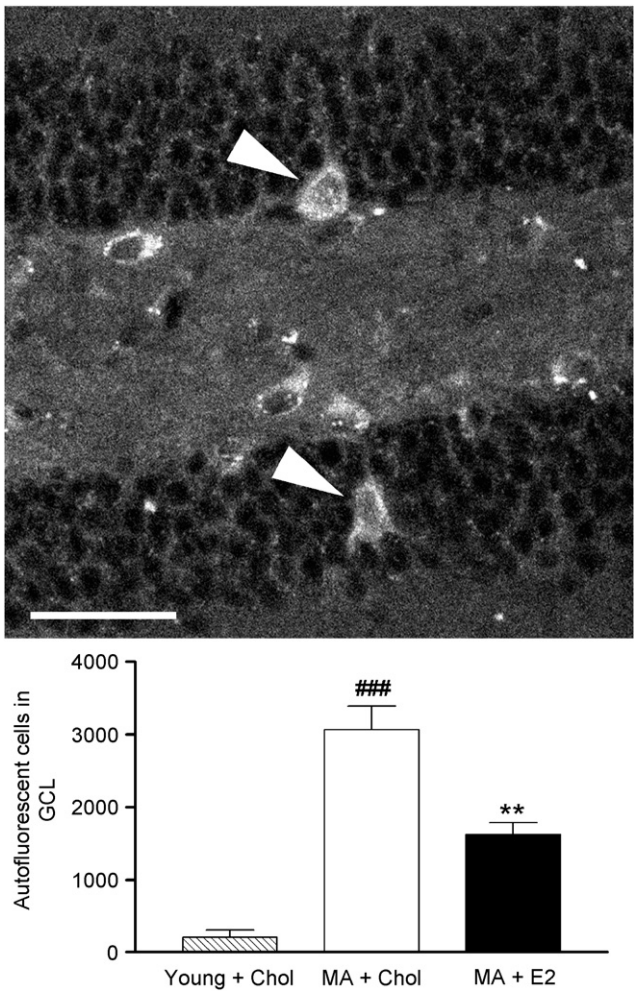


Figure 8 Lipofuscin autofluorescent cells in the GCL (arrowheads) from a MA chol-treated mouse. Bar: 50 μm. The bottom graph shows quantitative data from Exp. II. Young mice+chol correspond to the stippled column where few lipofuscin cells were found; MA+E_2-treated mice (dark column) presented significantly less lipofuscin-loaded cells than MA+chol mice (white column). *$p<0.05$ vs. MA+chol; ###$p<0.0001$ vs. Young+chol.

et al., 2001; McEwen et al., 2001; Behl, 2002). Finally, a role of estrogens on the modulation of the hypothalamic–pituitary–adrenal axis (HPA) may be an additional factor for the enhancement of DG cell proliferation caused by E_2. The hyperactive HPA axis of old animals (van Eekelen et al.,1991; Sapolsky, 1999; Dalm et al., 2005) can be attenuated by E_2 action at the hypothalamic and hippocampal levels (Ferrini et al., 1999; Isgor et al., 2003). Glucocorticoids inhibit hippocampal neurogenesis (Cameron and McKay, 1999; Kempermann et al., 2002; Miller and O'Callaghan, 2005) and E_2 could enhance neurogenesis by restraining the central stimulation of adrenal glucocorticoid secretion. Our preliminary data on the decreased plasma corticosterone of E_2-treated MA mice suggest this possibility.

Two additional neuronal effects resulted from E_2 treatment of MA mice. In one case, the high lipofuscin loading of neurons of the GCL characteristic of the aging brain, was markedly reduced by E_2. To understand its biological significance, it should be recall that lipofuscin deposits are aggregates of proteins, lipids and traces of carbohydrates (Keller et al., 2004). These aggregates result from aging-increased oxidative stress (Rivas-Arancibia et al., 2003). Therefore, disappearance of lipofuscin indicates that cells are relieved from this age-associated pathology, perhaps due to the anti-oxidant properties of estrogens (Behl, 2002). A third neuronal effect obtained in E_2-treated MA mice was the recovery of hilar neurons. Hilar neurons are a group of heterogenous neurons, some of them exhibiting a GABAergic phenotype and showing connections with the DG and other hippocampal fields (Mody et al., 1995). Their loss is observed after excitotoxic and ischemic injury suggesting an extreme vulnerability to oxidative agents (Azcoitia et al., 1998), which are greatly generated during aging. Therefore, it is tempting to hypothesize that recovery of hilar neurons represents another example of estrogen powerful antioxidant effects, a mechanism congruent with events proposed for lipofuscin unloading.

It is also feasible to establish a link between the effects of E_2 on neuronal proliferation and differentiation, to reactive astrocytosis. Reactive GFAP-expressing astrocytes are a hallmark of a number of brain pathologies, including aging (Goss et al., 1991; Nichols et al., 1993; David et al., 1994). The E_2 effect on GFAP+astrocytes in MA mice was expected on the grounds that this hormone down-regulated the reactive astrocytosis in the brain of old rats, young castrated rats, and animals with traumatic or excitotoxic lesions (Day et al., 1993; Garcia-Ovejero et al., 2002; Lei et al., 2003). These effects may be direct, because astrocytes express ER and an estrogen-response element exists on the 5′ upstream region of the GFAP promoter (Stone et al., 1998; Azcoitia et al., 1999). The reduced ability to manifest a GFAP reactive phenotype of the astrocytes of E_2-treated MA mice might prove beneficial for neurogenesis. In this respect, cell proliferation in the granular layer of the DG of 18 month old female mice is 48% higher in a $GFAP^{-/-}$ $Vimentin^{-/-}$ background, suggesting that genetic attenuation of age-associated reactive gliosis stimulates cell proliferation and neurogenesis (Larsson et al., 2004; Pekny and Nilsson, 2005). It was reported that E_2—even at physiological levels—inhibits GFAP expression in vivo and in vitro in a transcriptionally mediated manner (Rozovsky et al., 2002).

In conclusion, our experiments employing MA mice demonstrated that from the three basic steps of adult neurogenesis (proliferation, migration, and differentiation), E_2 stimulated progenitor proliferation and differentiation towards a neuronal lineage. This result, in conjunction with recovery from other aging indicators, supports a wide range protection of hippocampal function of MA mice by estrogenic hormones.

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Conflict of interest

The authors state that there are no actual or potential—including any financial or personal—conflicts of interest.

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