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Environmental enrichment attenuates the age-related decline in the mRNA expression of steroidogenic enzymes and reduces the methylation state of the steroid 5α-reductase type 1 gene in the rat hippocampus

María F. Rossetti [a,b], Jorgelina Varayoud [b], Guillermo S. Moreno-Piovano [b], Enrique H. Luque [b], Jorge G. Ramos [a,b,*]

[a] Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[b] Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral-CONICET, Santa Fe, Argentina

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ABSTRACT

We analyzed the effects of aging and environmental enrichment on the mRNA expression and DNA methylation state of steroidogenic enzymes in the hippocampus. The effects of aging were evaluated by comparing young adult (90-day-old) and middle-aged (450-day-old) female Wistar rats. To elucidate the effects of environmental enrichment, a subgroup of middle-aged rats exposed to sensory and social stimulation for 105 days was compared to rats housed under standard laboratory conditions. Aging decreased the transcription of neurosteroidogenic-related genes and increased the promoter methylation state of cytochrome P450 side chain cleavage, 3α-hydroxysteroid dehydrogenase (3α-HSD) and 5α-reductase-1. Exposure of middle-aged rats to environmental enrichment increased mRNA levels of 5α-reductase-1, 3α-HSD and cytochrome P450 17α-hydroxylase/c17,20-lyase and decreased the methylation state of the 5α-reductase-1 gene. Thus, sensory and social stimulation attenuate the age-related decline in the mRNA expression of hippocampal steroidogenic enzymes. Epigenetic mechanisms associated with differential promoter methylation could be involved.

1. Introduction

Aging is a physiological process that occurs in different areas of the brain and is associated with a decline in learning and memory functions (Bishop et al., 2010). Particularly in the cerebral cortex and hippocampus, changes have been described in various cellular, molecular and neural mechanisms that directly affect plasticity. In fact, several studies have shown changes in cell proliferation and neurogenesis (Klempin and Kempermann, 2007; Kuhn et al., 1996), dendritic branching (Grill and Riddle, 2002; Markham and Juraska, 2002), synaptic connectivity (Geinisman et al., 1992; Nicholson et al., 2004), Ca^{2+} homeostasis (Toescu and Verkhratsky, 2004; Toescu et al., 2004), neurotransmitter systems (Segovia et al., 2001), and the expression of neurotrophic factors (Hattiangady et al., 2005; Shetty et al., 2005), all of which alter the network dynamics of the neural ensembles that support cognition. Due to this neuronal vulnerability, the *incidence of neurodegenerative diseases*, such as Parkinson's disease and Alzheimer's disease (AD), *increases* dramatically with age (Mattson and Magnus, 2006).

Some authors have reported correlations between age-associated cognitive decline and decreased levels of steroids in women (Schumacher et al., 2003). Steroid hormones can be produced in the ovaries, adrenal glands and fat tissue; furthermore, neurosteroids can be synthesized de novo from cholesterol by both neurons and glial cells in various brain regions, including the hippocampus (Fig. 1) (Compagnone and Mellon, 2000; Reddy, 2010). Neurosteroids play several important roles associated with learning and memory (Charalampopoulos et al., 2008; Mellon, 2007; Reddy, 2010).

Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 17β-HSD-3, 17β-hydroxysteroid dehydrogenase type 3; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase; 5α-reductase, steroid 5α-reductase; 5α-reductase-1, steroid 5α-reductase type 1; AD, Alzheimer's disease; AP-1, activator protein 1; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding protein; Oct-1, octamer-binding factor-1; P450(11β)-1, 11β-hydroxylase; P450(11β)-2, aldosterone synthase; P450(17α), cytochrome P450 17α-hydroxylase/c17,20-lyase; P450(2d4), cytochrome P4502d4; P450arom, cytochrome P450arom; P450scc, cytochrome P450 side chain cleavage; Sp1, selective promoter factor 1; SREBP-, sterol regulatory element-binding protein; StAR, steroidogenic acute regulatory protein.

* Corresponding author. Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Casilla de Correo 242, 3000 Santa Fe, Argentina. Tel.: +54 342 4510283; fax: +54 342 4510283.
E-mail address: gramos@fbcb.unl.edu.ar (J.G. Ramos).

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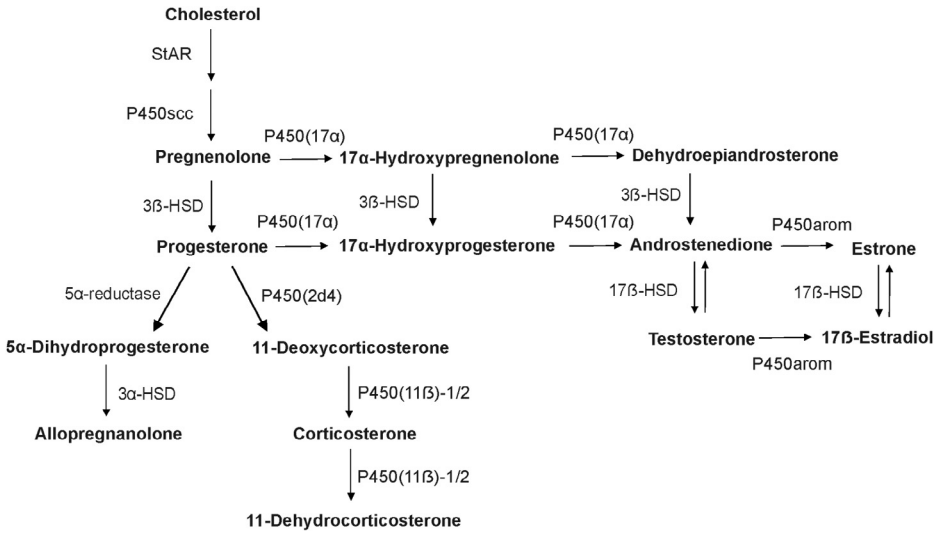

Fig. 1. Pathway of neurosteroid synthesis in the rat hippocampus. Steroidogenic acute regulatory protein (StAR); cytochrome P450 side chain cleavage (P450scc); 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD); cytochrome P450 17α-hydroxylase/c17,20-lyase (P450(17α)); steroid 5α-reductase (5α-reductase); 3α-hydroxysteroid dehydrogenase (3α-HSD); cytochrome. P4502d4 (P450(2d4)); 11β-hydroxylase (P450(11β)-1); aldosterone synthase (P450(11β)-2); 17β-hydroxysteroid dehydrogenase (17β-HSD) and cytochrome P450arom (P450arom).

Particularly, allopregnanolone promotes the proliferation of rodent neural progenitor cells and neuron survival (Wang et al., 2005), inhibits apoptosis in the late gestation fetal brain (Yawno et al., 2009), prevents memory impairment in rats (Escudero et al., 2012) and mice (Singh et al., 2012) and plays a key role in neurodegenerative diseases such as AD (Brinton, 2013; Irwin et al., 2011; Marx et al., 2006; Wang et al., 2008).

Several authors have provided new insight into neurosteroid synthesis and the association of decreasing neurosteroid levels with aging and various aging-related diseases (Charalampopoulos et al., 2006, 2008). In fact, the expression of certain enzymes involved in neurosteroidogenesis has been shown to change during neurodegenerative diseases (such as AD, Parkinson's disease and multiple sclerosis) and during aging (Higo et al., 2009; Kimoto et al., 2010; Luchetti et al., 2011a, 2011b). However, little is known about the molecular mechanism underlying these changes. One possibility is that steroidogenic enzyme expression could be affected by epigenetic mechanisms, such as DNA methylation and histone post-transcriptional modifications. Promoter hypermethylation has been reported for several genes during aging, including the estrogen receptor and insulin-like growth factor II (Calvanese et al., 2009). Moreover, Peleg et al. (2010) showed that memory disturbances in the brains of aging mice are associated with altered hippocampal chromatin plasticity. Importantly, histone deacetylase inhibition was also shown to ameliorate age-associated long-term memory impairment in 24-month-old rats, as assessed by the novel object recognition task (Reolon et al., 2011).

An enriched environment is "a combination of complex inanimate and social stimulation" (Rosenzweig et al., 1978) that improves learning and memory functions, even with aging and during neurodegenerative diseases (Frick and Fernandez, 2003; Laviola et al., 2008; van Praag et al., 2000). These experimental conditions are known to increase neural plasticity through changes in the morphology of the mammalian brain, such as neurogenesis, synaptic contacts and dendritic branching (Beauquis et al., 2010; Mora et al., 2007; Sale et al., 2009). Relationships between neurosteroidogenesis and animal housing conditions have been described. Social isolation has been shown to decrease the mRNA levels of the steroid 5α-reductase type 1 (5α-reductase-1) in the mouse hippocampus (Agis-Balboa et al., 2007); moreover, this condition increased the level of cytochrome P450arom (P450arom) mRNA and stimulated estradiol synthesis in the rat hippocampus (Munetsuna et al., 2009). In contrast, environmental enrichment increased the levels of mRNAs for 5α-reductase-1 and 3α-hydroxysteroid dehydrogenase (3α-HSD) in the hippocampus of young adult male rats (Munetsuna et al., 2011). Nonetheless, to the best of our knowledge, no prior reports have investigated the effects of environmental enrichment on the regulation of the mRNA expression of steroidogenic enzymes in the aged female rat hippocampus. Because there are several neurosteroids that exert vital neuronal functions, a comprehensive analysis is necessary to understand the effects of aging and environmental stimuli on steroidogenesis in the brain.

The purpose of the present study was to analyze the effects of aging and enriched environment on the mRNA expression of steroidogenic enzymes in the hippocampus. Moreover, we propose that epigenetic changes, such as differential DNA methylation, could be involved. These findings could contribute to the elucidation of the molecular mechanisms underlying the effects of aging on neurosteroid synthesis and the role of behavioral treatments as a mitigating factor.

2. Materials and methods

2.1. Animals

Female rats of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) with free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water supplied ad libitum in glass bottles with rubber stoppers surrounded by a steel ring. All rats were handled in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences and approved by the ethical committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral. Animals were treated humanely and with regard for the alleviation of suffering.

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2.2. Experimental design

Young adult (Y) and middle-aged (M) female rats with synchronous estrous cycles were used. Rats were maintained under standard laboratory conditions (SC) up to postnatal day (PND) 90 (Y-SC) or PND 345. At PND 345, middle-aged rats were divided into two groups. The first group of animals was exposed to an enriched environment (M-EE) until PND 450, and the second group was left in standard laboratory conditions (M-SC). The time course of the experiments is displayed in Fig. 2A.

Rats maintained under standard laboratory conditions were housed four per cage (41 cm long × 20 cm high × 30 cm wide) and were not exposed to enriching objects (Fig. 2B). Enriched rats were housed in a group of eight animals in large cages (80 cm long × 32 cm high × 51 cm wide) that were custom designed to provide social stimulation. Sensory stimulation was provided by an assortment of objects that always included large plastic tubes, rodent dwellings and toys of various shapes, sizes and colors (Fig. 2B). The rats were exposed to novelty stimulation by changing the exploratory objects daily, and care was taken not to include any individual object or toy more than once per week. Rats were enriched for 15 h daily from 18:00 to 09:00 h. The standard and enriched cages were maintained in the same colony room.

Young adult and middle-aged rats were sacrificed by decapitation at PND 90 or PND 450 according to the experimental design, and the hippocampus was quickly microdissected under a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY, USA), frozen in liquid nitrogen and kept at –80 °C for mRNA analysis and DNA methylation analysis.

2.3. Reverse transcription and real-time quantitative PCR analysis (qRT-PCR)

An optimized PCR protocol was employed to analyze the relative expression levels of steroidogenic molecules. The hippocampi of eight animals from each experimental group were individually homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A_{260}, and the samples were stored at –80 °C until later analysis. Equal quantities (4 µg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega, Madison, WI, USA) using 200 pmol of random primers (Promega, Madison, WI). Twenty units of ribonuclease inhibitor (RNAout) (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube at a final volume of 30 µl of 1× reverse transcriptase buffer. Reverse transcription was performed at 37 °C for 90 min and at 42 °C for 15 min. Reactions were stopped by heating at 80 °C for 5 min and cooling on ice.

Each reverse-transcribed product was diluted with RNAse free water to a final volume of 60 µl and further amplified in duplicate using the Real-Time DNA Step One Cycler (Applied Biosystems Inc., Foster City, CA, USA). Primer pairs used for the amplification of steroidogenic acute regulatory protein (StAR) (Munetsuna et al., 2011), cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD), cytochrome P450 17α-hydroxylase/c17,20-lyase (P450(17α)), 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD-3), P450arom, 5α-reductase-1, 3α-HSD (Munetsuna et al., 2011), aldosterone synthase (P450(11β)-2) and the ribosomal protein L19 (housekeeping gene) are shown in Table 1. For cDNA amplification, 5 µl of cDNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 µl. Each sample was quantified in duplicate or triplicate. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 52–60 °C for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, and these reactions did not yield any consistent amplification.

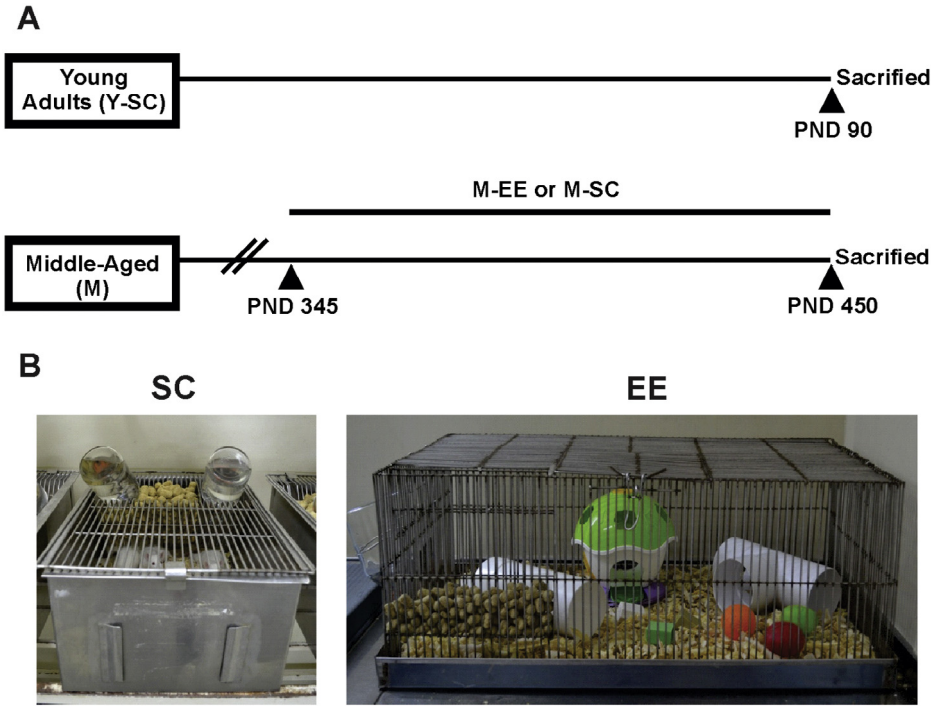

Fig. 2. Experimental protocol and caging conditions. (A) Rats were maintained under standard laboratory conditions (SC) from PND 0 to PND 90 (Y-SC) or to PND 450 (M-SC). At PND 345, a subgroup of rats was differentially housed in an enriched environment (M-EE) and was kept in these conditions up to PND 450 (105 days). (B) Experimental cages. In the left panel, a photograph of the standard caging (SC) condition is shown. The right panel shows the environmental enrichment (EE) condition.

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Table 1
The sequences of primer oligonucleotides for PCR amplification.

Targets	Primer sense	Primer antisense
StAR	5′-GCAAAGCGGTGTCAT CAG-3′	5′-GGCGAACTCTATCTGGG TCT-3′
P450scc	5′-AGGGAGAACGGCACACA CAG-3′	5′-TCGCAGGAGAAGAGAGT CGC-3′
3β-HSD	5′-CAGGGCATCTCTGTTGT CAT-3′	5′-AGATGAAGGCTGGCAC ACTA-3′
P450(17α)	5′-GGTGATAAAGGGTTATG CCA-3′	5′-GCTTGAATCAGAATGTC CGT-3′
17β-HSD-3	5′-CAACCTGCTCCCAAGTC ATT-3′	5′-AACCCCTACTCCCGAAG AAA-3′
P450arom	5′-TGGCAGATTCTTGTGGA TGG-3′	5′-CGAGGACTTGCTGATGAT GAGT-3′
5α-reductase-1	5′-CACCTTCAACGGCTATG TAC-3′	5′-AGGATGTGGTCTGAGTG GAT-3′
3α-HSD	5′-GCACTCAACTGGACTATGT GGA-3′	5′-GCTCATCTCGTGGGAAA AAT-3′
P450(11β)-2	5′-GAAGGTGCGTCAGAATG CTC-3′	5′-TTCAGGCTACCAGGGTT CAG-3′
L19 (housekeeping gene)	5′-AGCCTGTGACTGTCCAT TCC-3′	5′-TGGCAGTACCCTTCCTC TTC-3′

Table 2
The sequences of primer oligonucleotides for PCR amplification.

Targets	Primer sense	Primer antisense
IC P450scc	5′-ACCCATAAGGCAGACAT TGA-3′	5′-CCAAACGCAGAGAAAGA ACT-3′
Mae II (a) P450scc	5′-GAGACTTAATAGCAGTC CCA-3′	5′-GAGTAATAGCACACCCC TTT-3′
Mae II (b) P450scc	5′-GGAGGGGGTCCTAGCCA TTA-3′	5′-CCACTGCCCTTCAGACA GGT-3′
IC 3α-HSD	5′-CAGAGAAGGAAGTTTGA ATC-3′	5′-ATGTCAGATCACTTGGA AGT-3′
Mae II (a) 3α-HSD	5′-ACTGATTTTTGCTTAGG CTG-3′	5′-AAAATTCTGTAGTGAGC CGT-3′
Mae II (b) 3α-HSD	5′-GGATGTGGCTGGAATAC AGA-3′	5′-TTCTGTCACTTTGTCTG CCC-3′
Mae II (c) 3α-HSD	5′-GAAACATTGTGTCTGTA TGG-3′	5′-GTAAATTGTTAAGGGGA GAC-3′
IC 5α-reductase-1	5′-CAACTTTCTGTCCATCT ACC-3′	5′-CTTACAACTCTCCTCTT TCG-3′
BstUI (a) 5α-reductase-1	5′-CACCTTCCCAGCCCTGA CAG-3′	5′-AGGTGCCAGGAGAGAGG GGT-3′
Mae II (b) 5α-reductase-1	5′-AGTCAAGAAATATGCCT GAA-3′	5′-AATACGTTCTCGGTAT GAAT-3′
Mae II (c) 5α-reductase-1	5′-CCACTAAGCGTGAATCT CTC-3′	5′-AACACTCCATGACTCTC TGC-3′
Mae II (d) 5α-reductase-1	5′-CTGCTGGCTATGTTTCT GAT-3′	5′-TGGAATTAAGTCTCTGA GCC-3′

The relative expression levels of each target were calculated based on the cycle threshold (C_T) method (Higuchi et al., 1993). The C_T for each sample was calculated using the Step One Software (Applied Biosystems Inc. Foster City, CA, USA) with an automatic fluorescence threshold (*Rn*) setting. The efficiency of the PCR reactions for each target was assessed by the amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, the fold expression over control values was calculated for each target by the relative standard curve methods, which are designed to analyze data from real-time PCR (Cikos et al., 2007). For all experimental samples, the relative target quantity was determined from the standard curve, normalized to the relative quantity of the reference gene and finally divided by the normalized target value of the control sample. No significant differences in C_T values were observed for L19 among the various experimental groups.

2.4. Bioinformatics

The P450scc, 5α-reductase-1 and 3α-HSD rat genes (accession numbers AC_000076.1, AC_000069.1 and AC_000085.1, respectively) were analyzed for CpG islands using Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA). A CpG island was defined as a DNA sequence of 200 bp with a calculated percentage of CpGs of more than 50% and a calculated versus expected CpG distribution higher than 0.6. These regions were also checked for restriction sites for *BstU*I or *Mae II* enzymes to evaluate the number of methylation-sensitive sites. To recognize the putative binding sites for transcription factors, we used the TFSEARCH program. PCR primers were designed with Vector NTI Suite Version 6.0 software (Infomax Inc., North Bethesda, MD, USA) (Table 2).

2.5. Methylation-sensitive analysis

We investigated the methylation state of the P450scc, 3α-HSD and 5α-reductase-1 promoters in the experimental groups using a combination of digestions with methylation-sensitive restriction enzymes and subsequent real-time PCR analysis (Bruce et al., 2008; von Kanel et al., 2010). Hippocampal DNA from each group was individually prepared using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The concentration of total DNA was assessed by A_{260}, and DNA was stored at 2–8 °C until needed. Equal quantities (1.5 μg) of total DNA were digested with 7.5 units of BamHI (Promega, Madison, WI) to reduce the size of the DNA fragments and then purified with the Wizard SV gel and PCR Clean-Up System Kit (Promega, Madison, WI). A 130 ng sample of BamHI-cleaved DNA was digested overnight with 2 units of *BstUI* (New England BioLabs, Beverly, MA) or *Mae II* (Roche Applied Science, Indianapolis, IN) and 1X enzyme buffer at 60 °C or 50 °C, respectively, in a covered water bath (Tecno Dalvo, Santa Fe, Argentina) to ensure complete digestion. The digestion products were purified with the Wizard SV gel and PCR Clean-Up System Kit according to the manufacturer's protocol (Promega, Madison, WI). An optimized PCR protocol was employed to analyze the relative expression levels of various regions of the P450scc, 3α-HSD and 5α-reductase-1 promoters (Table 2). For DNA amplification, 5 μl of DNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 μl. Each sample was quantified in duplicate or triplicate. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 50–60 °C for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. The C_T for each sample and the PCR reaction efficiencies were calculated as described in Section 2.3. A region devoid of *BstU*I or *Mae II* restriction sites was amplified as an internal control (IC). When a CpG-rich site is methylated, enzymatic digestion with *BstU*I or *Mae II* is not possible, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *BstU*I or *Mae II* cleaves the DNA and prevents amplification of the fragment. The relative degree of promoter methylation was calculated by plotting C_T values against the log input (internal control), yielding standard curves for the quantification of unknown samples (Cikos et al., 2007).

2.6. Statistical analysis

Data (expressed as the mean ± SEM) were statistically analyzed by one-way ANOVA using the GraphPad Prism Version 5.03 statistical software package (GraphPad, San Diego, CA, USA). Post hoc multiple comparisons were made using Bonferroni's test. The methylation state of the sites corresponding to the P450scc promoter was compared using two tailed unpaired t-test. Differences were considered significant at $p < 0.05$.

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3. Results

3.1. Environmental enrichment attenuates, at least in part, the age-related decline in the mRNA expression of steroidogenic-related genes in the hippocampus of female rats

Most studies that have assessed the effect of aging on the mRNA expression of hippocampal steroidogenic enzymes/proteins were conducted in male rats at the ages of PND 1 (neonatal rats) to PND 90 (adult rats) (Higo et al., 2009; Ibanez et al., 2003; Kimoto et al., 2010). In contrast, our study was performed using young adult and middle-aged female rats. Our results showed that the gene expression of StAR, P450scc, 3β-HSD, P450arom, P450(17α), 5α-reductase-1, 3α-HSD, P450(11β)-2 and 17β-HSD-3 decreased by at least 2-fold in the M-SC rats ($p < 0.05$; Fig. 3) compared to the Y-SC group. In contrast, long-term environmental enrichment was able to attenuate the decline in expression for these genes, increasing the gene expression of StAR (1.25-fold; $p < 0.05$), 3α-HSD (1.26-fold; $p < 0.05$), 5α-reductase-1 (1.2-fold; $p < 0.05$) and P450(17α) (1.38-fold; $p < 0.05$) in aged rats (M-EE versus M-SC; Fig. 3). However, the mRNA levels of these genes were still significantly lower than the levels in young adult animals (M-EE versus Y-SC; $p < 0.05$; Fig. 3).

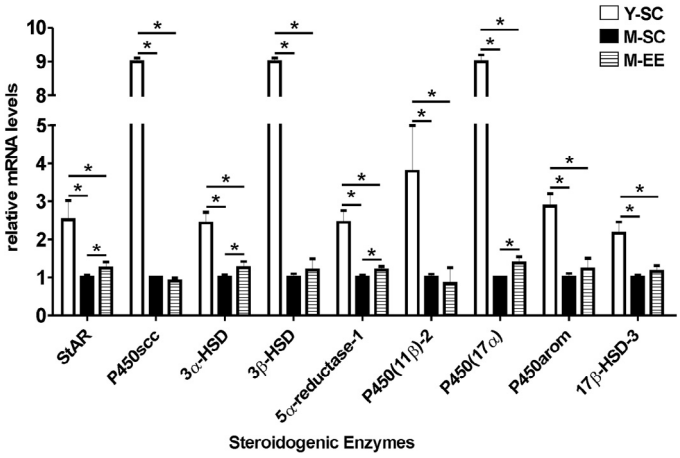

Fig. 3. Real-time PCR analysis of the mRNA levels of StAR and steroidogenic enzymes in the female hippocampus of young adult (Y-SC) and middle-aged rats housed under standard laboratory conditions (M-SC) and of middle-aged rats exposed to an enriched environment for 105 days (M-EE). The amounts of mRNA in the Y-SC and M-EE groups are presented as relative values versus those of the M-SC group. The columns and error bars represent the means ± SEM. * indicates a significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA.

3.2. In silico analysis of candidate sites of DNA methylation and potential transcription binding sites in the rat P450scc, 5α-reductase-1 and 3α-HSD genes

Based on these results and with a particular interest in allopregnanolone synthesis, we decided to study whether the P450scc, 5α-reductase-1 and 3α-HSD genes in rodents are epigenetically regulated. Accordingly, we analyzed their promoter regions and searched for candidate sites for DNA methylation. The results are shown in Fig. 4.

3.3. Aging and environmental enrichment modify the DNA methylation pattern of certain steroidogenic-related genes

To determine if the changes observed in the P450scc, 5α-reductase-1 and 3α-HSD transcript levels are related to DNA methylation modifications, we determined the methylation state of

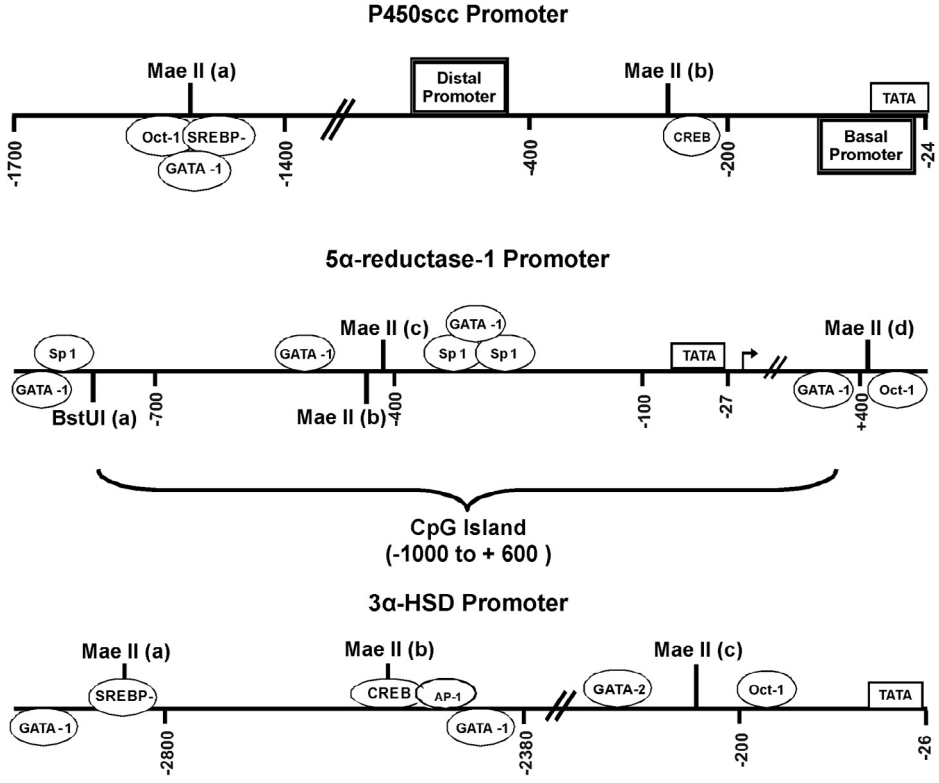

Fig. 4. Maps of the P450scc (Shih et al., 2011), 5α-reductase-1 (Blanchard et al., 2007) and 3α-HSD (Penning, 1996) promoters, their binding proteins and methylation-targeted CG areas. The positions of the TATA box are indicated. Predicted binding sites for transcription factor cAMP response element-binding protein (CREB), octamer-binding factor-1 (Oct-1), GATA, sterol regulatory element-binding protein (SREBP-), selective promoter factor 1 (Sp1) and activator protein 1 (AP-1) are shown as circles above the line. CpG islands and CG target sites for digestion by the methylation-sensitive restriction enzymes *BstUI* (CGCG) and *Mae II* (ACGT) are indicated.

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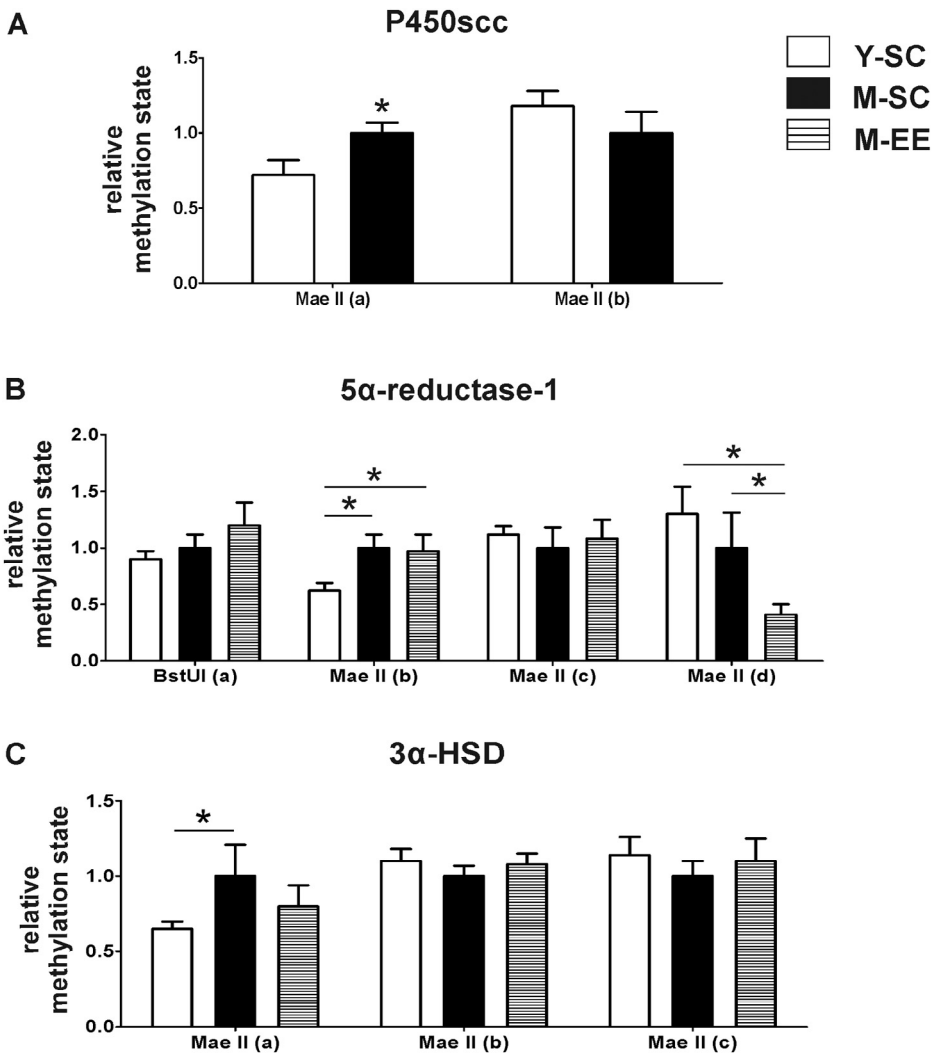

Fig. 5. Methylation analysis using methylation-sensitive restriction enzymes followed by real-time PCR in the female hippocampus of young adult (Y-SC) and middle-aged rats housed under standard laboratory conditions (M-SC) and middle-aged rats exposed to environmental enrichment for 105 days (M-EE). Methylation-sensitive restriction sites of the P450scc (A), 5α-reductase-1(B) and 3α-HSD (C) gene promoters were studied. The relative methylation state in the Y-SC and M-EE groups are indicated as relative values versus those of the M-SC group. The columns and error bars represent the means ± SEM. * indicates a significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA or by two-tailed unpaired t-test, as appropriate .

the transcriptionally active promoters of these enzymes in the Y-SC, M-SC and M-EE groups. Genomic DNA extracted from the hippocampus was incubated with the *Mae II* and *BstUI* restriction enzymes, and the targeted DNA regions were studied by real-time PCR. Non-digested DNA served as a control for DNA quality. An internal control (included within the promoter region) was used as a control for quantitative PCR analysis.

In the P450scc promoter, an increase in the methylation state was detected at the *Mae II* site (a) in M-SC rats compared to Y-SC rats ($p < 0.05$, Fig. 5). Because environmental enrichment did not affect P450scc mRNA expression (Fig. 3), no methylation-sensitive analysis was conducted in the M-EE group.

In the 5α-reductase-1 promoter, the methylation state of the *Mae II* site (b) increased in the M-SC and M-EE groups compared to the young adult rats ($p < 0.05$, Fig. 5). When we analyzed the 5α-reductase-1 exon 1 region, the methylation state at the *Mae II* site (d) was significantly decreased in the M-EE rats compared to the M-SC and Y-SC groups ($p < 0.05$, Fig. 5).

In the 3α-HSD promoter region, the methylation state at the *Mae II* site (a) increased in aged compared to young adult rats ($p < 0.05$, Fig. 5). No changes were detected at other methylation-targeted CG sites.

4. Discussion

Aging is a process that degrades brain plasticity, favoring general neuronal dysfunction (Bishop et al., 2010). Conversely, environmental enrichment promotes neuronal protection through various mechanisms of action (Beauquis et al., 2010; Mora et al., 2007; Sale et al., 2009). Interactions between the negative effects of aging and the positive plasticity processes associated with environmental enrichment can restore specific cognitive or motor behaviors (Mora et al., 2007). In addition, neurosteroids have positive effects on neurogenesis, synaptic connectivity and cognitive performance (Charalampopoulos et al., 2008; Mellon, 2007; Reddy, 2010).

In the present study, we evaluated the effects of aging and an enriched environment on the mRNA expression of hippocampal neurosteroidogenic molecules. We hypothesized that epigenetic modifications may be involved in these processes. Given the importance of allopregnanolone synthesis, our study focused on three relevant involved enzymes: P450scc, 5α-reductase-1 and 3α-HSD. To the best of our knowledge, this is the first study to (1) establish a relationship between the decline in the mRNA expression of these steroidogenic enzymes with age and changes in promoter

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methylation state in the female hippocampus and (2) report an attenuation of this decline in response to prolonged exposure to an enriched environment; in the case of the 5α-reductase-1 gene, these effects occurred through differential DNA methylation mechanisms.

A decrease in the mRNA expression of certain hippocampal enzymes involved in the synthesis of steroids such as P450scc, P450arom, P450(17α) and 3β-HSD has been previously described, although the majority of these studies have been conducted in neonatal and adult male rats (Higo et al., 2009; Ibanez et al., 2003; Kimoto et al., 2010). Here, we provided the first demonstration of an important decrease in the transcription of hippocampal steroidogenic-related genes in female rats between PND 90 and PND 450. While the mRNA expression of StAR, P450arom, 5α-reductase-1, 3α-HSD, P450(11β)-2 and 17β-HSD-3 was 2.5- to 3.8-fold higher in young adult rats than in aged rats, the expression of the 3β-HSD, P450(17α), and P450scc genes was 9-fold higher in young adult rats compared to aged rats. Interestingly, Dong et al. (2001) previously correlated an approximately 2-fold decrease in the levels of 5α-reductase protein and mRNA with a 50% reduction in allopregnanolone levels in the frontal cortex of socially isolated mice compared to group-housed mice. In addition, several authors have shown that the inhibition of a particular steroidogenic enzyme can affect the synthesis of certain neurosteroids. As an example, the administration of 5α-reductase inhibitors, such as (17β)17[[bis(1-methylethyl) amino]carbonyl] androstane-3,5-diene-3-carboxylic acid and Finasterine, have been reported to decrease the levels of allopregnanolone in the male rat brain (Cheney et al., 1995; Mukai et al., 2008). In this context, our results suggest that the major changes found in steroidogenic enzyme mRNA expression in the aged female hippocampus could be associated with the alteration of neurosteroid levels during postnatal development.

Based on these results, we studied whether the P450scc, 5α-reductase-1 and 3α-HSD genes are epigenetically regulated. We analyzed the potential sites for transcription factor binding and DNA methylation in silico, and we analyzed the methylation state of the promoters of these three enzymes in vitro using methylation-sensitive restriction analysis. In aged rats, we observed hypermethylation at the P450scc, 5α-reductase-1 and 3α-HSD promoters, which may explain the decreased mRNA expression of these enzymes. Interestingly, we found that the P450scc promoter was mostly methylated at a potential binding site for the SREBP-, Oct-1 and GATA-1 transcription factors. GATA family proteins have been suggested to have a role in the regulation of P450scc transcription in the placenta and the ovary (Sher et al., 2007; Shih et al., 2011). In addition, SREBP- and Oct-1 have been shown to regulate the expression of certain steroidogenic molecules, such as StAR (Lavoie and King, 2009) and 3α-HSD (Penning, 1996). Similarly, a potential binding site for SREBP- was predicted in the mostly methylated site within the 3α-HSD promoter. The fact that these elements act as regulators of steroidogenic enzyme expression supports the idea that these methylation-sensitive sites could be potential transcriptional regulatory sites.

Differential methylation has been shown to regulate steroidogenesis. Modifications in the methylation state of the steroidogenic enzyme genes P450scc and 3β-HSD have been previously described (Vanselow et al., 2010; Zhang and Ho, 2011). In addition, changes in the DNA methylation of the P450(17α) gene were observed during cellular senescence in bovine adrenocortical cells. A methylated CpG island was identified in the P450(17α) promoter within the rat adrenal gland (Missaghian et al., 2009). Another study demonstrated that the P450arom promoter 2 is regulated by DNA methylation in bovine granulose cells and corpora lutea (Vanselow et al., 2005). The responsiveness of the P450arom promoter to cAMP also seems to be regulated by CpG methylation (Demura and Bulun, 2008). According to our results, P450scc, 5α-reductase-1 and 3α-HSD promoter methylation was also found to be inversely correlated with enzyme gene expression in the hippocampus of aged female rats. These results suggest that the negative effects of aging could be mediated by a decline in age-related neurosteroidogenic enzymes, which is regulated, at least in part, by DNA methylation mechanisms. Thus, treatments that reverse the hypermethylation of these genes may attenuate the decline of neurosteroid levels in adulthood, promoting hippocampal neuronal plasticity.

It is well known that environmental enrichment increases neural plasticity and is one of the most reliable and well-characterized paradigms of experience-dependent plasticity in rodents (Munetsuna et al., 2011), although the underlying mechanism remains unclear. In this context, it would be interesting to know whether environmental stimuli could attenuate the decline in the mRNA expression of neurosteroidogenic molecules during aging. Here, we found that long-term environmental enrichment increased the expression of StAR, 5α-reductase-1, 3α-HSD and P450(17α) mRNA in middle-aged female rats. Munetsuna et al. (2011) also showed that long-term environmental enrichment increased the mRNA levels of 5α-reductase-1 (1.2-fold) and 3α-HSD (2.8-fold) in the hippocampus of male young adult rats. In our experiment, the mRNA levels of steroidogenic-related genes increased only 1.2- to 1.4-fold in the enriched rats, and these levels were still significantly lower compared to those in the young adult rats. Nevertheless, small changes in mRNA enzyme expression have been shown to affect steroid metabolism. Higo et al. (2009) showed that the rate of metabolism for androgens and estrogen was higher in the hippocampus of PND10 male rats than in adults, although the mRNA expression of several steroidogenic-related enzymes (e.g., 3β-HSD, P450arom, P450(17α) and 17β-HSD) was only 1.3- to 1.5-fold higher in the hippocampus at PND10 than at adulthood. Thus, it could be possible that these small, but significant, changes observed in the gene expression of steroidogenic molecules in response to environmental enrichment attenuated the age-related decline of allopregnanolone synthesis, at least in part, in the hippocampus of female rats.

Various types of stimuli can induce epigenetic modifications in certain genes. Weaver et al. (2004) found that maternal care alters the DNA methylation pattern of exon 1_7 of the glucocorticoid receptor promoter sequence in the adult offspring. In addition, Kuzumaki et al. (2011) showed that the induction of brain-derived neurotrophic factor (BDNF) expression is correlated with significant changes in histone methylation in the hippocampus of mice exposed to an enriched environment. Along the same line, we found changes in the methylation pattern of the 5α-reductase-1 gene in the hippocampus of enriched rats; these changes were correlated with an increase in mRNA expression. Contrary, no changes in the methylation state of the 3α-HSD gene were found in the hippocampus. However, due to the limitations of the technique, some methylation-targeted CG sites were not included in the analysis. It is also possible that the transcription of this gene is regulated by other mechanisms that were not included in this study. Histone modifications have been associated with transcriptional repression or the activity of genes involved in steroid hormone biosynthesis and action (Martinez-Arguelles and Papadopoulos, 2010). Moreover, some authors have examined the implications of certain transcription factors in the regulation of 3α-HSD gene expression. The 5′-flanking regions of the rat and human genes contain consensus sequences for AP-1, Oct-1 and steroid hormone response elements, which may comprise a steroid response unit (Lin and Penning, 1995). In this context, Penning (1996) provided evidence that glucocorticoid response elements and Oct factors act as positive and negative regulators of the transcription of this gene, respectively. In addition, it was suggested that the *trans*-acting factors involved in increasing gene expression may include steroid hormone receptors and members of the AP-1 transcription factor family (Lin and Penning, 1995). Thus, it is an ongoing challenge to study other possible epigenetic modifications or alterations in the action of transcriptions factors that are associated with the regulation of the mRNA expression of the 3α-HSD gene.

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Young rats, which have higher allopregnanolone levels in the cortex (proestrous and late pregnancy), have been shown to exhibit better performance on the object recognition task than diestrous rats or rats in early pregnancy (Frye, 2009). Similarly, other studies have indicated that allopregnanolone can improve memory impairment and inhibit the symptoms of aging and neurodegenerative diseases. Escudero et al. (2012) showed that treatment with estradiol benzoate alone or with progesterone in ovariectomized rats has amnesic effects and that allopregnanolone can reverse this effect, suggesting that the beneficial effects may include enhancement of the cognitive performance of the hippocampus. In patients with Alzheimer's disease, reduced allopregnanolone levels were observed in the prefrontal cortex (Marx et al., 2006). Wang et al. (2010) demonstrated that allopregnanolone reversed neurogenic and cognitive deficits in a mouse model of AD. In addition, Singh et al. (2012) provided preclinical evidence that allopregnanolone promotes the survival of newly generated cells and restores cognitive performance in a mouse transgenic model of AD as well as in normal aging. Moreover, the administration of allopregnanolone in a mouse model of the human neurodegenerative disease Niemann–Pick disease type C increased Purkinje and granule cell survival (Griffin et al., 2004). Our results suggest that the improvements in memory and learning functions induced by an enriched environment could be mediated, at least in part, by demethylation mechanisms, which cause an increase in the mRNA expression of certain enzymes involved in the synthesis of allopregnanolone.

5. Conclusions

The present study demonstrated that the mRNA expression of the genes involved in hippocampal steroid synthesis diminished with age in female rats. Moreover, the hypermethylated state of the P450scc, 5α-reductase 1 and 3α-HSD promoters detected in aged rats suggests epigenetic control of mRNA expression. In contrast, environmental enrichment was able to increase the gene expression of certain steroidogenic molecules in aged animals. In particular, we detected an increase in the mRNA expression of two enzymes responsible for allopregnanolone synthesis, 5α-reductase-1 and 3α-HSD. In addition, a reduction in the relative methylation state of the 5α-reductase-1 gene was found. Thus, enrichment could help to maintain adequate levels of neurosteroidogenic enzymes, potentially promoting hippocampal neuronal plasticity and preventing age-related neurodegenerative diseases.

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