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Pregnancy and lactation differentially modify the transcriptional regulation of steroidogenic enzymes through DNA methylation mechanisms in the hippocampus of aged rats

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ABSTRACT

In the present study, we examined the mRNA expression and DNA methylation state of steroidogenic enzymes in the hippocampus of young adult (90-days-old) and middle-aged (450-days-old) nulliparous rats, and middle-aged multiparous rats subjected to three pregnancies with and without lactation. Aging decreased the mRNA levels of steroidogenic-related genes, while pregnancy and lactation significantly reduced the effect of aging, maintaining high expression levels of cytochrome P450 side-chain cleavage (P450scc), steroid 5α-reductase-1 (5αR-1), cytochrome P450arom (P450arom) and aldosterone synthase (P450(11β)-2). In addition, pregnancy and lactation diminished the methylation state of the 5αR-1 promoter and increased the transcription of brain-derived neurotrophic factor, synaptophysin and spinophilin. Pregnancy without lactation increased P450scc and 5αR-1 gene expression and decreased the methylation of their promoters. We concluded that the age-related decrease in the mRNA expression of steroidogenic enzymes is differentially attenuated by pregnancy and lactation in the rat hippocampus and that differential methylation mechanisms could be involved.

1. Introduction

The aging process is accompanied by relevant physiological and structural changes in the brain that include alterations in synaptic connectivity (Geinisman et al., 1992; Nicholson et al., 2004), neurogenesis (Klempin and Kempermann, 2007; Kuhn et al., 1996), dendritic branching (Grill and Riddle, 2002; Markham and Juraska, 2002) and dynamics of several neurotransmitters (Segovia et al., 2001). Deficits in the gene expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor and insulin-like growth factor type 1 have also been described (Hattiangady et al., 2005; Shetty et al., 2005). Importantly, there is evidence that aging has an important role in the occurrence of neurodegenerative diseases such as Alzheimer's (AD), Parkinson's and Huntington's disease (Mattson and Magnus, 2006).

In the last years, some of these changes have been associated with disparities in steroid and neurosteroid levels during aging (Charalampopoulos et al., 2006, 2008). Neurosteroids are synthesized *de novo* from cholesterol in the nervous system by both neurons and glial cells of various brain regions (Fig.1), including the hippocampus (Compagnone and Mellon, 2000; Reddy, 2010), and play different important roles associated with learning and

Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ;5-Δ;4-isomerase; 5αR, steroid 5α-reductase; Ad, activating domain; AD, Alzheimer's disease; AP-3, activator protein 3; BDNF, brain-derived neurotrophic factor; CLS, cAMP-responsive element-like sequence; NRE, nuclear receptor element; 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; PI.f, brain promoter; PI.tr, testis promoter; PII, ovary and testis promoter; StAR, steroidogenic acute regulatory protein; syp, synaptophysin.

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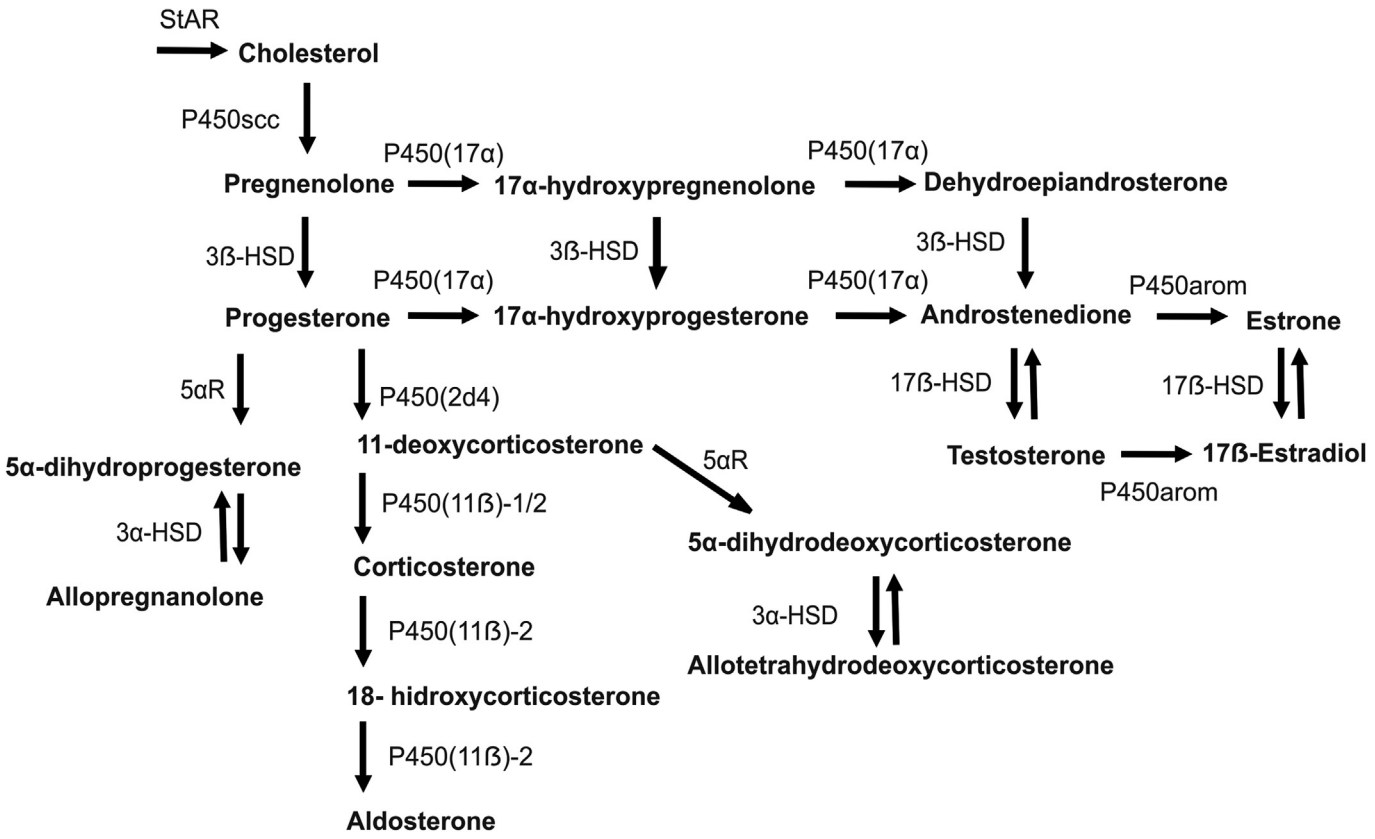

Fig. 1. Pathway of neurosteroid synthesis in the rat hippocampus. Steroidogenic acute regulatory protein (StAR); cytochrome P450 side-chain cleavage (P450scc); 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD); cytochrome P450 17α-hydroxylase/c17,20-lyase (P450(17α)); steroid 5α-reductase (5αR);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

memory functions (Charalampopoulos et al., 2008; Mellon, 2007; Reddy, 2010). In fact, it has been shown that neurosteroids enhance neuronal survival and recovery in aged animals and in different lesion paradigms. Dai et al. (2007) showed that estradiol rescues the synaptic transmission and long-term potentiation induction impaired after transient global ischemia. Estradiol also protects neurons against β-amyloid peptide toxicity in culture and promotes neurogenesis within the adult hippocampal formation (Schumacher et al., 2003). Progesterone and allopregnanolone exert marked neuroprotective effects after spinal cord injury and traumatic brain injury (Garcia-Ovejero et al., 2014; Guennoun et al., 2015; He et al., 2004; Stein, 2008). Progesterone also plays an important role in the formation and repair of myelin sheaths, which are necessary for the efficient and rapid conduction of action potentials (Charalampopoulos et al., 2008). In addition, it has been reported that allopregnanolone plays a key role in maintaining cognitive abilities during neurodegenerative diseases such as AD (Singh et al., 2012; Wang et al., 2010).

Some authors have shown that the expression of certain neurosteroidogenic enzymes change with progressive age (Higo et al., 2009; Kimoto et al., 2010; Luchetti et al., 2011a, 2011b), which may explain the decrease in steroid levels in the brain during postnatal development (Charalampopoulos et al., 2006). However, little is known about the molecular mechanisms involved. Recently, we correlated a decrease in the mRNA expression of the hippocampal steroidogenic enzymes cytochrome P450 side-chain cleavage (P450scc), steroid 5α-reductase type 1 (5αR-1) and 3α-hydroxysteroid dehydrogenase (3α-HSD) with an increase in the methylation state of their promoters in aged rats (Rossetti et al., 2015). DNA methylation is defined as the reversible addition of methyl groups to the 5-position of cytosine within DNA strands and it is one of the most studied mechanisms for silencing gene expression (Rodenhiser and Mann, 2006). Thus, it is possible that the reversal of the hypermethylation age-associated state of steroidogenic-related genes may attenuate the decrease in neurosteroid levels in adulthood, potentially promoting hippocampal neuronal functions.

Maternal experience and its accompanying exposure to various reproductive hormones such as prolactin, progesterone and estrogen, produce physiological changes that include modifications in various cellular, molecular and neural mechanisms. In fact, maternal experience has been shown to have a long-lasting impact on learning and memory abilities, as well as on neuroplasticity functions associated with the hippocampus (Franssen et al., 2012; Gatewood et al., 2005; Kinsley et al., 2006). Particularly, it has been shown that multiparous rats have better performance on spatial (Morris water maze) and non-spatial (Object recognition) memory tests than nulliparous rats and show an attenuation of age-associated cognitive impairment. In addition, reproductive experience decreases deposits of the deleterious amyloid precursor protein, a marker of neurodegeneration, and increases the expression of BDNF, which acts as a nerve growth factor, in the rat hippocampus (Gatewood et al., 2005; Macbeth et al., 2008). Changes in neurogenesis and spine density in the CA1 area of the hippocampus have also been described (Vanoye-Carlo et al., 2008). Moreover, Franssen et al. (2012) suggested that the plasticity of the maternal brain may facilitate neural and behavioral recovery from kainic acid-induced neural insult.

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Importantly, some of these effects have been observed a long time after the last reproductive event.

Considering that maternal experience and neurosteroids have positive effects on neuronal and cognitive functions, it is possible that the beneficial effects of reproduction on the brain are related to the enhancement of neurosteroid biosynthesis. To our knowledge, no studies have assessed the long-lasting impact of pregnancy and lactation on steroidogenesis in the aged brain. The purpose of the present study was to analyze the effect of multiparity and lactation on the age-related decrease in mRNA neurosteroidogenic enzyme expression, and to evaluate whether differential DNA methylation mechanisms are implicated. Thus, we analyzed the levels of mRNA and the DNA methylation state of multiple genes involved in steroidogenesis in the hippocampus of young adult and middle-aged nulliparous rats and middle-aged multiparous rats subjected to three pregnancies with and without their respective lactation. Additionally, we evaluated the mRNA expression of BDNF, synaptophysin (syp) and spinophilin as molecular markers of neurotrophic and synaptic hippocampal functions.

2. Materials and methods

2.1. Animals and experimental design

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, Santa Fe, Argentina. Animals were treated humanely and with regard for alleviation of suffering. Female rats were obtained from timed-pregnant rats of an inbred Wistar-derived strain bred at the Department of Human Physiology of the Universidad Nacional del Litoral. From conception until the end of the experiment, rats were housed under controlled conditions (23 ± 2 °C and 12 h light–dark cycle) 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. At 3 months of age, female rats were randomly divided into four groups (n = 8/group): 1) young adult nulliparous (YN; no pregnancies); 2) middle-aged nulliparous (AN; no pregnancies); 3) middle-aged multiparous subjected to three pregnancies with their respective lactation (AM + L) and 4) middle-aged multiparous subjected to three pregnancies without lactation (AM-L). Multiparous rats were subjected to their pregnancies at post-natal day (PND) 90, PND 160 and PND 230. For the AM-L group, pups were removed from their mother's cages on PND 0. The time course of the experiments is displayed in Fig. 2.

Young adult and middle-aged rats were sacrificed by decapitation on PND 90 or PND 450, according to the experimental design. Importantly, all animals were sacrificed during the diestrous phase of the estrous cycle in order to maintain similar endocrine conditions (Beale et al., 2014). No significant differences in estrous cycle were observed in experimental groups (Fig. 1; Supplementary Data). Middle-aged animals were sacrificed at least 3 months after the last weaning, considering the acute effect of reproduction on the levels of certain neurosteroids such as allopregnanolone (Concas et al., 1998). Brain tissue blocks containing the hippocampus were quickly microdissected under a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY, USA). The hippocampus was immediately removed and frozen in liquid nitrogen and kept at −80 °C for mRNA analysis and DNA methylation analysis.

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

An optimized PCR protocol was used to analyze the relative expression levels of steroidogenic molecules. The hippocampi of

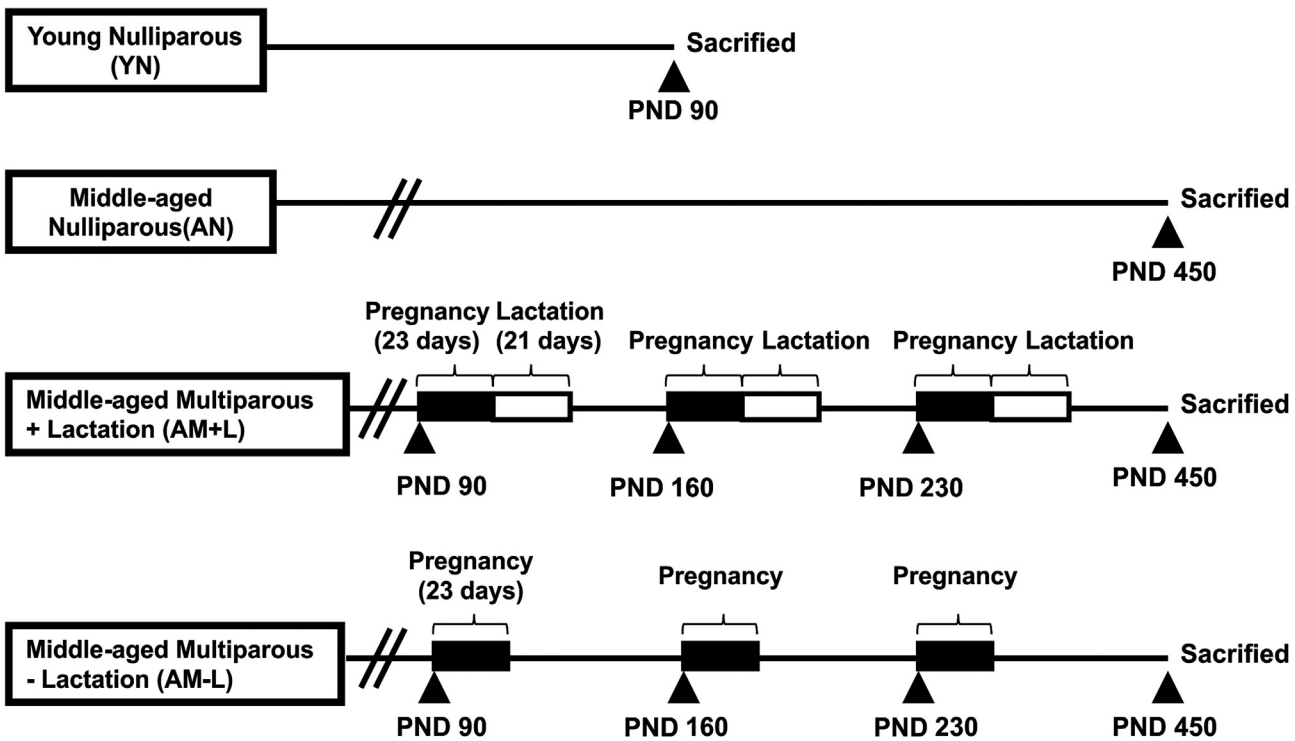

Fig. 2. Experimental protocol. Young adult (YN) and middle-aged (AN) nulliparous female rats were sacrificed on PND 90 and PND 450 respectively. Multiparous female rats subjected to three pregnancies with (AM + L) and without (AM-L) their respective lactation at PND 90, PND 160 and PND 230 were sacrificed on PND 450.

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Table 1
Sequences of primer oligonucleotides for PCR amplification.

Target gen	Primer sense	Primer antisense
BDNF	5′-GACTCTGGAGAGCGTGAATG - 3′	5′-GAACCTTCTGGTCCTCATCC - 3′
Syp	5′- TGTTTGCCTTCCTCTACTCC - 3′	5′- CCCAGGCTGATGAACTAACT - 3′
Spinophilin	5′- GGAATCAGGGTGTGTGGAGA - 3′	5′- GCGTCGGTCATAGTCCTCAT - 3′

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), as reported in Rossetti et al. (2015).

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), 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), cytochrome P450arom (P450arom), 5αR-1, 3α-HSD, aldosterone synthase (P450(11β)-2), and the ribosomal protein L19 (housekeeping gene) were previously described in Rossetti et al. (2015). Primer pairs used for the amplification of BDNF, syp and spinophilin are described 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) 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. 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.) with an automatic fluorescence threshold (*Rn*) setting. The efficiency of PCR reactions was assessed for each target 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.

2.3. Bioinformatics

The P450arom and P450 (11β)-2 gene promoters described by Stocco (2008) and Nomura et al. (1993) were analyzed for CpG islands using the Methyl Primer Express Software v1.0 (Applied Biosystems). 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 *BstUI* (New England BioLabs, Beverly, MA, USA) or *Mae II* (Roche Applied Science, Indianapolis, IN, USA) to evaluate the number of methylation-sensitive sites. PCR primers were designed with the software Vector NTI Suite Version 6.0 (Table 2).

2.4. Methylation-sensitive analysis

We investigated the methylation state of the P450scc, 5αR-1, P450arom and P450(11β)-2 promoters, 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 (n = 8/group) was individually prepared using the Wizard Genomic DNA Purification Kit (Promega). 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) to reduce the size of the DNA fragments and then purified with the Wizard SV gel and PCR Clean-Up System Kit (Promega). A 130-ng sample of BamHI-cleaved DNA was digested overnight with 2 units of *BstUI* or *Mae II* 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).

An optimized PCR protocol was used to analyze the relative expression levels of various regions of the P450scc, 5αR-1, P450arom and P450(11β)-2 gene promoters. The P450scc and 5αR-1 promoter regions have been previously studied and the PCR primers for amplification have been described in Rossetti et al. (2015); primer pairs for P450arom and P450(11β)-2 are detailed in Table 2. For DNA amplification, 5 μl of DNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica) and 10 pmol of each primer (Invitrogen) 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. The product purity was confirmed by dissociation curves, and random

Table 2
Sequences of primer oligonucleotides for PCR amplification.

Target gen	Primer sense	Primer antisense
IC P450arom	5′- ACTCAAGGGCAAGATGATAA- 3′	5′- AGTGACAAGGCAAGACAAAT- 3′
Mae II P450arom	5′- CTGGAGTAGGAGCCTTTACC- 3′	5′- CTTGAGTGGGTAGAGTGACG- 3′
IC P450(11β)-2	5′-TGGACACTACACCTGCTCTT- 3′	5′-GGGCTCAACTGTCAGTAGAA- 3′
Mae II P450(11β)-2	5′-GAGCCCCAACCATGACCAGA- 3′	5′-TGCCATCCTCCTCCCTTCAG- 3′

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samples were subjected to agarose gel electrophoresis. The C_T for each sample and the PCR reaction efficiencies were calculated as described in Section 2.2. A region devoid of *BstU*I and *Mae II* restriction sites was amplified as an internal control. 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 the amplification of the fragment. The relative degree of promoter methylation was calculated by C_t values plotted against the log input (internal control), yielding standard curves for the quantification of unknown samples (Cikos et al., 2007).

2.5. Statistical analysis

An exploratory analysis was conducted first to confirm the normal distribution of the data (Shapiro–Wilk test) and variance homogeneity (Levene's test). Data (expressed as the means ± SEM) were statistically analyzed by one-way ANOVA using GraphPad Prism Version 5.03 statistical software package (GraphPad, San Diego, CA, USA). Post-hoc multiple comparisons were made using Bonferroni's test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. The age-related decrease in mRNA levels of hippocampal steroidogenic enzymes is differentially attenuated by pregnancy and lactation

By using quantitative real-time PCR, we studied the mRNA expression of hippocampal steroidogenic enzymes and StAR in young adult and middle-aged nulliparous female rats (YN and AN, respectively) and multiparous rats subjected to three pregnancies with their respective lactations (AM + L) and without them (AM-L). One-way ANOVA revealed differences in the transcription of StAR (p:0.0007, $F_{(3,28)}$:7.974), P450scc ($p < 0.0001$, $F_{(3,28)}$:32.77), 3β-HSD ($p < 0.0001$, $F_{(3,28)}$:41.53), P450(17α) ($p < 0.0001$, $F_{(3,28)}$:38), 5αR-1 ($p < 0.0007$, $F_{(3,28)}$:14.34), 3α-HSD (p:0.0014, $F_{(3,28)}$:6.817), P450(11β)-2 (p:0.041, $F_{(3,28)}$:5.529), 17β-HSD-3 ($p < 0.0001$, $F_{(3,28)}$:23.49) and P450arom ($p < 0.0001$, $F_{(3,28)}$:15.56) across all the experimental groups. The Bonferroni post-test showed that the mRNA expression of steroidogenic-related genes decreased by at least 2-fold in AN rats compared to the YN group ($p < 0.05$; Fig. 3). Interestingly, the AM + L group increased the transcription of the P450scc (2.7-fold, $p < 0.05$) and 5αR-1 (1.57-fold, $p < 0.05$) genes compared to the AN group (Fig. 3). In the same way, the AM-L rats showed an increase in the mRNA levels of the P450scc (1.39-fold versus AN, $p < 0.05$) and 5αR-1 genes (1.53-fold versus AN, $p < 0.05$). The gene expression of P450arom was higher in AM + L (1.85-fold, $p < 0.05$) and was lower in AM-L (0.4-fold, $p < 0.05$) than in AN rats. The transcription of P450(11β)-2 gene also increased in AM + L (2.9-fold, $p < 0.05$) and decreased in the AM-L (0.18-fold, $p < 0.05$) compared to the AN group. When we compared multiparous rats with and without lactation, we found that the mRNA expression of P450scc, P450arom and P450(11β)-2 was higher in AM + L than in AM-L by at least 2-fold ($p < 0.05$; Fig. 3). However, the mRNA levels of these genes were still significantly lower in AM + L and AM-L than in YN ($p < 0.05$; Fig 3); only the AM + L group reversed the age-related decrease in the mRNA expression of P450(11β)-2 (AM + L versus YN; $p > 0.05$).

3.2. In silico analysis of candidate sites of DNA methylation in the rat P450arom and P450(11β)-2 gene promoters

Based on the results, we decided to study whether the P450scc, 5αR-1, P450arom and P450(11β)-2 genes in rats are epigenetically regulated. The P450scc and 5αR-1 promoter regions, their predicted binding proteins and methylation-targeted CG areas have been previously described (Rossetti et al., 2015). Here, we analyzed the

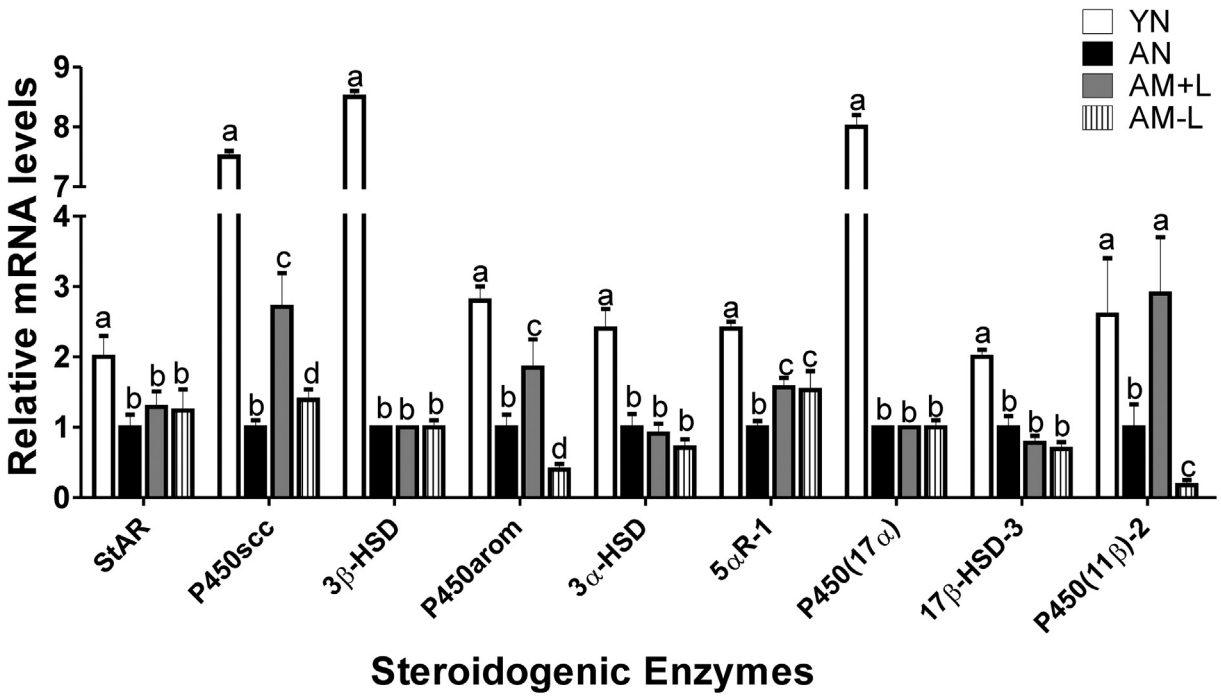

Fig. 3. Real-time PCR analysis of the mRNA levels of StAR and steroidogenic enzymes in the hippocampus of young adult (YN) and middle-aged (AN) nulliparous female rats and multiparous rats with (AM + L) and without (AM-L) their respective lactation. The amounts of mRNA in YN, AM + L and AM-L are indicated as relative values versus those of AN. The columns and error bars represent the means ± SEM (n = 8/group). The mRNA expression of each gene was analyzed independently. Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA. Column bars with the same letter were not significantly different.

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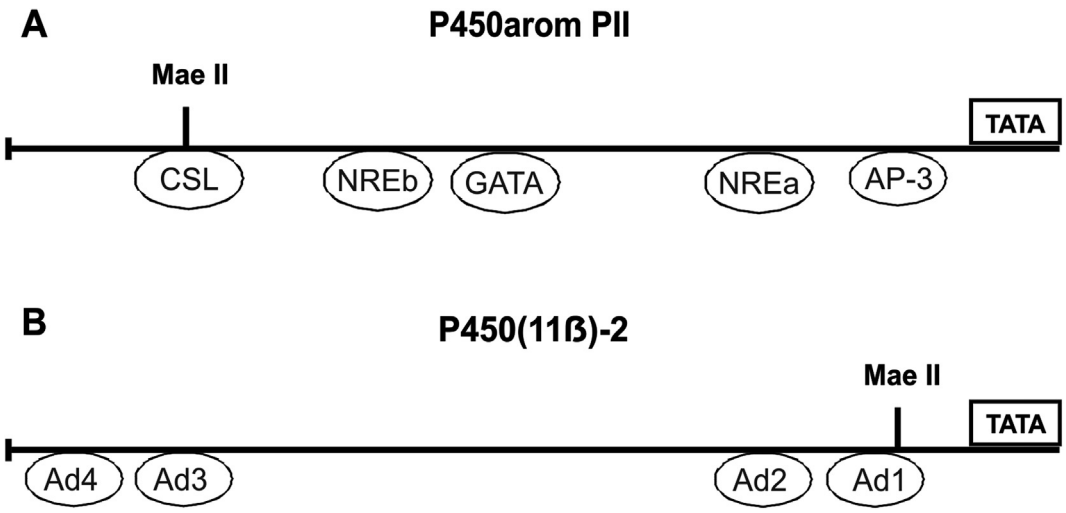

Fig. 4. Maps of P450arom (A) (Stocco, 2008) and P450(11β)-2 (B) (Nomura et al., 1993) promoters, their binding proteins and methylation-targeted CG areas. The positions of the TATA box are indicated. Predicted binding sites for transcription factor cAMP-responsive element-like sequence (CLS), activator protein 3 (AP-3), nuclear receptor elements a and b (NREa and NREb), GATA and activating domains (Ad1-4) and CG target sites for digestion by the methylation-sensitive restriction enzyme *Mae II* (ACGT) are indicated.

promoter regions of the P450arom and P450(11β)-2 genes and searched for candidate sites for DNA methylation. In the rat, three P450arom promoters have been described: PI.f (brain promoter), PII (ovary and testis promoter) and PI.tr (testis promoter) (Silandre et al., 2007). We detected that both the brain and the ovarian transcripts are produced in the rat hippocampus. Based on that, we analyzed PI.f and PII looking for methylation-targeted CG areas. As no target sites were detected in PI.f (data not shown), only PII was included in this study.

PII promoter was described previously by Stocco (2008). This region includes the TATA box, a cAMP-responsive element-like sequence (CLS), two binding sites for members of the nuclear receptors 5A family of transcription factors (NREa and NREb), one response element for members of the zinc finger family of transcription factors known as GATA and one activator protein (AP) 3 binding site. Although no CpG Island was found in the rat P450arom promoter region, an isolated CG site was presented that could be a potential site for methylation and, in consequences, target for digestion by the methylation sensitive restriction enzyme *Mae II*. Interestingly, this restriction site corresponds to the CLS sequence, supporting the idea that it could be a possible regulatory site of gene expression. The results are shown in Fig. 4A.

On the other hand, P450(11β)-2 promoter region was also described (Nomura et al., 1993). The 5′ upstream region of this rat gene contains the TATA box and four cis-*acting* elements Ad1, a cAMP response element homolog, Ad2, Ad3 and Ad4 (Fig. 4B). No CpG Island was found in this promoter region; however, we found an isolated CG site that could be a potential site for differential methylation activity and a target for digestion by the methylation sensitive restriction enzyme *Mae II* (Fig. 4B).

3.3. Pregnancy and lactation generate long-term changes in the methylation state of the P450scc, 5αR-1 and P450arom genes in the rat hippocampus

To evaluate whether pregnancy and lactation-induced changes in the transcript expression of P450scc, 5αR-1, P450arom and P450(11β)-2 were associated with differential DNA methylation, we determined the methylation state of the transcriptionally active promoters of these enzymes. One-way ANOVA showed differences in the methylation levels of *Mae II a* (p:0.0012, $F_{(3,28)}$:6.959) and b ($p < 0.0001$, $F_{(3,28)}$:27.01) sites within P450scc promoter, *Mae* II b ($p < 0.0001$, $F_{(3,28)}$:15.71), c (p:0.0012, $F_{(3,28)}$:7.78) and d ($p < 0.0001$, $F_{(3,28)}$:55.96) sites within 5α-reductase-1 promoter and *Mae* II site ($p < 0.0001$, $F_{(3,28)}$:211.8) from P450arom promoter; thus, a Bonferroni post-test was performed. In the P450scc promoter, an increase in the methylation state was detected at the *Mae II* site (a) in AN compared with the YN and AM-L groups (AN: 1 ± 0.07 versus YN: 0.7 ± 0.12 and AM-L: 0.54 ± 0.08, $p < 0.05$, Fig. 5A). In addition, the methylation state of *Mae II* site (b) was higher in AN and YN rats than in AM-L ones (AN: 1 ± 0.14 and YN: 1.22 ± 0.10 versus AM-L: 0.71 ± 0.10, $p < 0.05$, Fig. 5A). In the 5αR-1 promoter, the methylation state of *Mae II* site (b) was higher in AN rats than in YN and AM + L ones (AN: 1 ± 0.12 versus YN: 0.62 ± 0.07 and AM + L: 0.39 ± 0.13, $p < 0.05$, Fig. 5B). Moreover, an increase in the methylation state of this site was detected in AM-L compared with AM + L (AM + L: 0.39 ± 0.1 versus AM-L: 1 ± 0.13, $p < 0.05$, Fig. 5B), while an opposite effect was detected at the *Mae II* site (c) (AM + L: 1.10 ± 0.16 versus AM-L: 0.71 ± 0.16, $p < 0.05$, Fig. 5B). When we analyzed the 5αR-1 exon 1 region, the methylation state at the *Mae II* site (d) was significantly lower in AM-L rats than in YN, AN and AM + L ones (AM-L: 0.12 ± 0.03 versus AN: 1 ± 0.31, YN: 1.30 ± 0.24 and AM + L: 1.24 ± 0.29, $p < 0.05$, Fig. 5B). In the P450arom promoter, an increase in the methylation state was detected at the *Mae II* site in AM-L compared with YN, AN and AM + L (AM-L: 2.61 ± 0.34 versus AN: 1 ± 0.18, YN: 1.26 ± 0.23 and AM + L: 1 ± 0.13, $p < 0.05$, Fig. 5C). No changes were detected at other methylation-targeted CG sites.

3.4. Pregnancy and lactation increase the mRNA levels of hippocampal BDNF, syp and spinophilin genes

To analyze the effects of maternal experience on neuronal functions, we evaluated the mRNA expression of neurotrophic and synaptic markers in YN, AN, AM + L and AM-L rats. The comparison between groups using one-way ANOVA exposed differences in the mRNA levels of BDNF (p:0.0300, $F_{(3,28)}$:3.521), syp (p:0.0006, $F_{(3,28)}$:7.777) and spinophilin (p:0.0408, $F_{(3,28)}$:3.144). The Bonferroni post-test showed that aging decreased the gene expression of BDNF (YN: 1.50 ± 0.15 versus AN: 1 ± 0.16, $p < 0.05$) and syp (YN: 1.40 ± 0.10 versus AN: 1 ± 0.01, $p < 0.05$) in the hippocampus of female rats (Fig. 6). Interestingly, pregnancy and lactation

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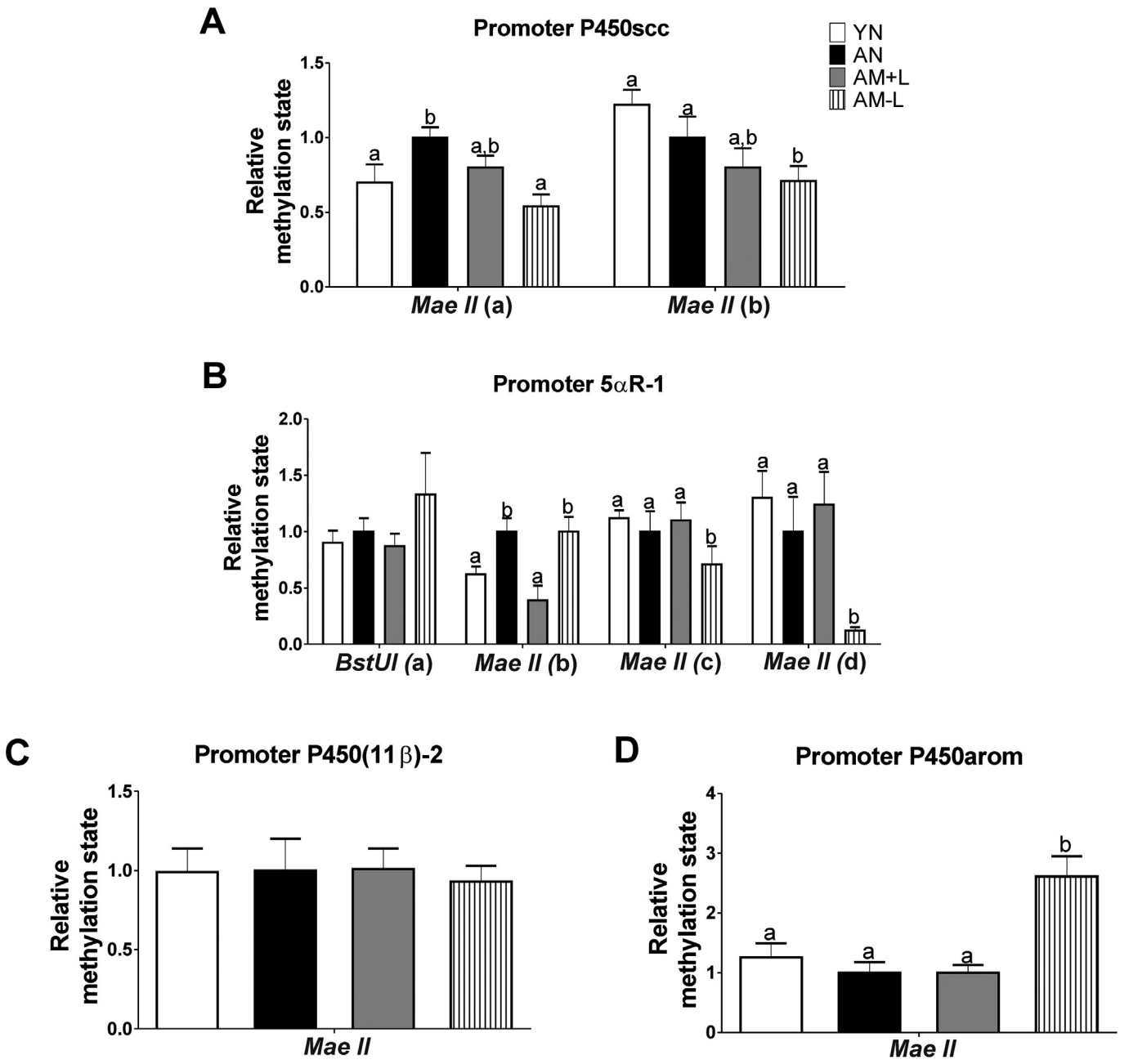

Fig. 5. Methylation analysis using methylation-sensitive restriction enzymes followed by real-time PCR in the hippocampus of young adult (YN) and middle-aged (AN) nulliparous female rats and multiparous rats with (AM + L) and without (AM-L) their respective lactation. Methylation analysis was performed for the P450scc (A), 5αR-1 (B), P450arom (C) and P450(11β)-2 (D) gene promoters. The relative methylation states in YN, AM + L and AM−L are indicated as relative values versus those of AN. The columns and error bars represent the means ± SEM (n = 8/group). The methylation levels of each methylation-sensitive restriction enzyme site were analyzed independently. Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA. Column bars with the same letter were not significantly different.

maintained a greater transcription rate of BDNF (1.41-fold, $p < 0.05$), syp (1.24-fold, $p < 0.05$) and spinophilin (1.42-fold, $p < 0.05$), when AM + L animals were compared with the AN group, reversing the age-related decline of neurotrophic gene expression (Fig. 6).

4. Discussion

The current study compared the mRNA expression of steroidogenic-related genes in the hippocampus of young adult and middle-aged nulliparous rats and multiparous middle-aged rats subjected to three pregnancies with and without the lactation periods. In addition, the DNA methylation state of P450scc, 5αR-1, P450arom and P450(11β)-2 promoters was analyzed. Moreover, the effect of pregnancy and lactation on the transcription of neurotrophic and synaptic genes was evaluated. To our knowledge, this is the first study to report that pregnancy and lactation differentially (1) attenuate the age-related decrease in the mRNA expression of certain steroidogenic enzymes and (2) modify the methylation state of the P450scc, 5αR-1 and P450arom promoters in the female rat hippocampus.

The plasma levels of several reproductive hormones, such as estradiol, progesterone and prolactin, significantly increase during reproduction (Amenomori et al., 1970; Higuchi et al., 1985; Taya and

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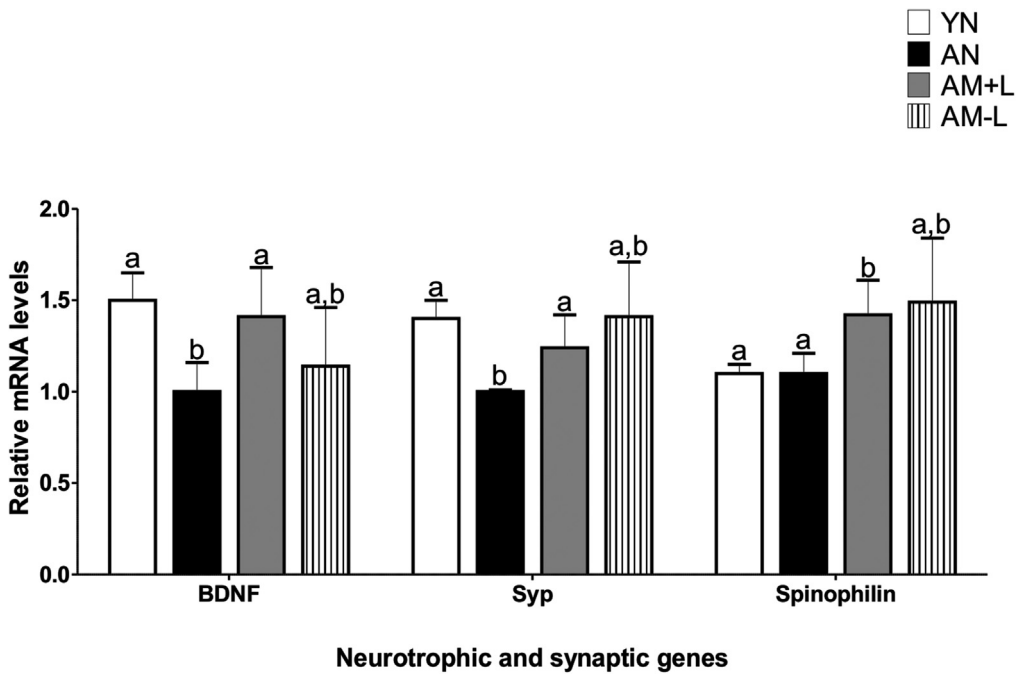

Fig. 6. Real-time PCR analysis of the mRNA levels of BDNF, syp and spinophilin in the hippocampus of young adult (YN) and middle-aged (AN) nulliparous female rats and multiparous rats with (AM + L) and without (AM-L) their respective lactation. The amounts of mRNA in YN, AM + L and AM-L are indicated as relative values versus those of AN. The columns and error bars represent the means ± SEM (n = 8/group). The mRNA expression of each gene was analyzed independently. Different letters indicate significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA. Column bars with the same letter were not significantly different.

Greenwald, 1981). Interestingly, a large number of animal studies also support the beneficial effects of these hormones on the functioning and viability of neurons and on learning and memory processes (Leuner et al., 2012; Morales et al., 2014; Schumacher et al., 2003). Moreover, several authors have reported a correlation between these steroid hormones and the regulation of neurosteroidogenesis (Bixo et al., 1997; Frye et al., 2000; Kato et al., 2013). Particularly, estradiol modifies the mRNA expression of 3β-HSD and 3α-HSD in the rat hypothalamus (Soma et al., 2005) and hippocampus (Mitev et al., 2003), respectively. Estradiol also increases progesterone levels in the rat hypothalamus (Soma et al., 2005). Interestingly, Sanchez et al. (2008) demonstrated that the mRNA levels of both 5αR isozymes are significantly increased by sulpiride in the brain of rats, either directly or via sulpiride-induced hyperprolactinemia. Reproductive hormones also play significant roles in the conversion from indifference and nonresponsiveness to rapid-onset maternal behavior (Bridges, 1984; Carter, 2003; Grattan et al., 2001). The interactions between a mother and her offspring (pup's care, feeding and protection) during labor and lactation are also an important and complex stimulus. In fact, Kinsley and Lambert (2008) suggested that the interaction of reproductive hormones with postpartum pup exposure produces a kind of 'enriched environment' since it implicates a combination of complex social, cognitive and sensorimotor stimuli (Lambert et al., 2005). Recently, our research group has reported an increase in the mRNA expression of the steroidogenic genes StAR, 5αR-1, 3α-HSD and P450(17α) in the hippocampus of female rats exposed to environmental sensory stimuli (Rossetti et al., 2015). We also found that this change in gene expression is accompanied by a modification in the methylation pattern of 5αR-1 (Rossetti et al., 2015). Based on the above, we propose that the successive exposures to reproductive hormones including the lactation period or not (without mother − pup interaction) regulate the mRNA expression of certain steroidogenic enzymes in the rat hippocampus.

Our results showed that pregnancy increased the transcription of P450scc and 5αR-1 but decreased the mRNA expression of P450arom and P450(11β)-2 in the hippocampus of aged rats. Although the removal of the offspring on PND 0 from maternal cages could have generated a stress condition in the mother, this is the model that has been previously used when studying pregnancy as an isolated process (Zhong et al., 1990). P450scc is involved in the first step of steroid synthesis and limits the metabolism rate, whereas 5αR is related to allopregnanolone synthesis (Do Rego et al., 2009). Despite the fact that the changes in mRNA expression do not always reflect changes in protein abundance, the correlation between mRNA and protein levels of steroidogenic enzymes was previously described. Particularly, an approximately 2-fold decrease was found in 5αR-1 mRNA and protein levels with a 50% reduction in allopregnanolone synthesis in the frontal cortex of socially isolated mice compared to group-housed mice (Dong et al., 2001). Thus, it is possible that the increase found in the mRNA expression of this enzyme in the hippocampus of multiparous rats is associated with alterations in the protein levels and, in consequence, in the synthesis of the mentioned steroid. In fact, it is known that mating induces neurosteroidogenesis of allopregnanolone in the midbrain, cortex and striatum, as well as in the hippocampus (Frye, 2009). Moreover, allopregnanolone reaches maximal levels on day 19–20 of pregnancy and decreases just prior to parturition in the rat brain (Concas et al., 1998). In this context, we propose that successive reproductive experiences could increase the levels of allopregnanolone in the rat hippocampus by the transcriptional activation of 5αR-1 and that this effect could persist even for a long time after the last reproductive event, as we report in this work.

The beneficial effects of lactation have been described in different animal models (Russo and Russo, 1980; Yang et al., 1999). In fact, Morales (2011) showed that lactation is a natural model for neuroprotection since it effectively prevents acute and chronic cell damage of the hippocampus induced by excitotoxicity. Changes in dendritic branching (Kinsley et al., 2006; Pawluski and Galea,

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2006), synaptic plasticity (Tomizawa et al., 2003) and cell proliferation (Leuner et al., 2007) in the hippocampus have also been described. In the same direction, we found that lactation is necessary for the transcriptional activation of the principal enzymes involved in estradiol and corticosterone synthesis and to maintain the effects of pregnancy in increasing the mRNA expression of the enzyme implicated in allopregnanolone synthesis in the rat hippocampus. In addition, lactation also increased the gene expression of BDNF, syp and spinophilin, which are proteins involved in synaptic functions, neurogenesis, dendritic spine formation and cognition (Binder and Scharfman, 2004; Croll et al., 1998; Feng et al., 2000; Kwon and Chapman, 2011; Sze et al., 1997). Moreover, the modification of BDNF and syp levels have been described during normal aging and AD and this fact was correlated with cognitive decline and other neuronal dysfunctions (Heinonen et al., 1995; Liu et al., 2005; Sze et al., 1997; Tapia-Arancibia et al., 2008). Contrary, some physiologic or pathologic age-associated changes in the nervous system could be offset by the administration of exogenous BDNF (Tapia-Arancibia et al., 2008). Thus, lactation could improve synaptic and cognitive functions in the adult rat hippocampus, as was previously described by several authors (Gatewood et al., 2005; Kinsley et al., 2006; Kinsley and Lambert, 2008), enhancing the expression of specific neuronal related-genes such as BDNF, syp and spinophilin. *In vivo* and *in vitro* studies have shown that neurotrophic and synaptic gene expression in the rodent hippocampus is regulated by certain steroid hormones such as progesterone (Aguirre and Baudry, 2009; Younan et al., 2012), estrogens (Frick et al., 2002; Kretz et al., 2004; Lee et al., 2004; Moreno-Piovano et al., 2014; Rune et al., 2002) and corticosterone (Hansson et al., 2006). All the above suggests that an increase in the transcription of steroidogenic genes could improve hippocampal neurosteroid levels, which enhance the expression of specific neuronal genes. Moreover, some authors have described that rats and mice exposed to sensory, motor and/or cognitive stimulation also modify the gene expression of BDNF and syp in the hippocampus (Ickes et al., 2000; Lambert et al., 2005). Consequently, it is possible that both changes in neurosteroid levels and maternal enrichment are responsible for inducing the improvement in the expression of these hippocampal genes.

To explain the changes found in the mRNA expression of steroidogenic enzymes in multiparous rats, we analyzed the methylation patterns of their promoter regions. Our results showed that the methylation state of the 5αR-1 gene was decreased by both conditions, pregnancy with and without lactation, although two different regulatory sites were affected in each case. Interestingly, we have previously reported changes in the methylation state of these sites caused by aging and enriched environments (Rossetti et al., 2015). This suggests that these changes could be involved in the transcriptional regulation of this gene. In the present study, pregnancy without lactation also decreased the methylation levels of the P450scc promoter, but increased P450arom gene methylation. One of the sites of the P450scc promoter that changed its methylation pattern is a potential binding site for the sterol regulatory element-binding protein, octamer-binding factor-1 and GATA-1 transcription factors, as previously described (24). This, added to the fact that the methylation levels of the same site are also affected by aging (Rossetti et al., 2015), supports the idea that it could be a possible regulatory site of gene expression. On the other hand, a cAMP-responsive element-like sequence (CLS) has been described in the mostly methylated site within the P450arom promoter II (Stocco, 2008). This region is recognized by the cAMP-responsive element binding protein and the mutation of this element greatly reduces the induction of promoter activity by cAMP (Fitzpatrick and Richards, 1994; Michael et al., 1997). CLS is also recognized by the transcription factor CCAAT/enhancer-binding protein beta, which has an inhibitory effect on aromatase promoter activity in human endometrial cells (Yang et al., 2002). Thus, the hypermethylation of this site could be inhibiting P450arom expression in the hippocampus of multiparous rats. In contrast, we found no changes in the methylation state of the P450 (11β)-2 gene. Due to the limitations of the technique, some methylation-targeted CG sites were not included in the analysis; however, it is also possible that the transcription of this gene is regulated by other epigenetic mechanisms that were not included in this study, such as histone modifications (Martinez-Arguelles and Papadopoulos, 2010). In addition, the implications of certain transcription factors in the regulation of P450(11β)-2 expression have also been reported (Bassett et al., 2004).

Neurosteroids play important roles in brain function. Estradiol increases neuronal survival and recovery and promotes neurogenesis in adult animals (Schumacher et al., 2003). It is also essential for synaptic plasticity and modulates the expression of synaptic proteins such as syp and spinophilin (Rune and Frotscher, 2005). Allopregnanolone prevents memory impairment in rats (Escudero et al., 2012), promotes the proliferation of rodent neural progenitor cells (Wang et al., 2005) and inhibits apoptosis (Yawno et al., 2009). It also promotes the survival of newly generated cells and restores cognitive performance in a mouse transgenic model of AD as well as in normal aging (Singh et al., 2012). Corticosterone as a neurosteroid has been much less studied than estradiol and allopregnanolone. However, it is known that glucocorticoids are involved in memory formation (Lupien and Lepage, 2001) and some authors have reported that corticosteroids enhance memory consolidation in the rodent hippocampus (Cottrell and Nakajima, 1977; Kelemen et al., 2014; Micheau et al., 1984). In this context, we proposed that the improvements caused by pregnancy and lactation in neuronal and cognitive functions may be mediated, at least in part, by demethylation mechanisms, which cause an increase in the mRNA expression of different enzymes involved in the synthesis of allopregnanolone, estradiol and corticosterone in the hippocampus.

5. Conclusions

The present study demonstrated that reproductive experience attenuates the age-related decrease in the mRNA expression of the genes involved in hippocampal steroid synthesis in female rats, but that pregnancy and lactation have a differential effect on it. The combination of both activated the transcription of P450scc, 5αR-1, P450arom and P450(11β)-2, enzymes involved in the synthesis of allopregnanolone, estradiol and corticosterone, and increased the mRNA levels of neurotrophic and synaptic factors. In contrast, pregnancy without lactation increased the gene expression of 5αR-1, but decreased the mRNA expression of enzymes related to estradiol and corticosterone synthesis. In addition, changes in the DNA methylation pattern of the P450scc, 5αR-1 and P450arom promoters in the hippocampus of multiparous rats suggest an epigenetic control of mRNA expression of steroidogenic-related genes. Thus, pregnancy and lactation could help to maintain adequate levels of certain neurosteroids in adulthood, potentially improving cognition and neuronal functions and preventing neurodegenerative diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.03.037.

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