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Sex- and age-associated differences in episodic-like memory and transcriptional regulation of hippocampal steroidogenic enzymes in rats

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ABSTRACT

The aim of this study was to evaluate the episodic-like memory (ELM) and the transcriptional regulation of the enzymes involved in hippocampal allopregnanolone synthesis in young adult and middle-aged male and female rats. Young adult males, but not middle-aged ones, showed a good performance in the ELM task. In contrast, neither young nor middle-aged females were able to discriminate the spatial order in which the objects were presented. In females, aging decreased the transcription of steroidogenic-related genes. In addition, the mRNA levels of 5α-reductase-1 were higher and the methylation of its promoter was lower in young adult females than in males, suggesting an epigenetic control. Further studies are needed to establish correlations between ELM and the transcriptional regulation of hippocampal steroidogenic enzymes. Our results contribute to the knowledge of sex differences in gene expression, methylation and memory during aging.

1. Introduction

The evidence of sex differences in the brain and behavior has been growing in the last years. Studies in rodents have shown variations in tests of depression- and anxiety-like symptoms, response to drugs of abuse, response to stress and learning and memory functions (locomotor activity, social interaction, spatial memory, and object recognition). In addition, previous studies have shown sexual dimorphism in the levels of monoamine neurotransmitters (e.g. hippocampal dopamine), levels of neurotrophic factors (e.g. brain-derived neurotrophic factor), neurogenesis and plasticity (Ngun et al., 2011; Simpson and Kelly, 2012). Understanding the sex-specific differences in neurobiology and behavior is necessary to better understand basic aging mechanisms. In female and male rats, aging involves similar neurochemical and structural variations in the hippocampus and the cerebral cortex, such as alterations in dendritic branching (Grill and Riddle, 2002), synaptic connectivity (Nicholson et al., 2004) and neurotransmitter systems (Segovia et al., 2001). This neuronal vulnerability causes a deterioration of the hippocampal-dependent memory functions such as object recognition, spatial memory and episodic-like memory (ELM) (Pause et al., 2013; Tulving, 2002; Wallace et al., 2007). ELM refers to the ability to remember personal experience in terms of what happened, and when and where it happened, and is extremely sensitive to normal brain aging and neurodegenerative diseases, turning it a key indicator of incipient neurological disease (Binder et al., 2015; Dere et al., 2005). However, there are no prior reports of the potential differential effect of aging on this kind of memory in female and male rodents.

Hippocampal cognitive decline has been previously related to decreased levels of steroids (Tuscher et al., 2015). Steroid hormones can be produced in the ovaries, adrenal glands and fat tissue (Schumacher et al., 2003), and it is now well known that they are also synthesized *de novo* from cholesterol in numerous brain regions, by both neurons and glia. These locally synthesized

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hormones are called neurosteroids (Schumacher et al., 2003). One of the most well studied neurosteroids is allopregnanolone, which plays a key role in memory (Escudero et al., 2012; Singh et al., 2012) and in neurodegenerative diseases (Irwin et al., 2011; Marx et al., 2006). Fig. 1 depicts the pathway of allopregnanolone synthesis in the rat hippocampus. The first enzymatic reaction of this pathway is rate-limiting and involves the transformation of cholesterol into pregnenolone, catalyzed by the P450 cholesterol side-chain cleavage enzyme (P450scc), which is located in the inner mitochondrial membrane. Cholesterol supply to this membrane is mainly mediated by steroidogenic acute regulatory protein (StAR), which is located in the outer mitochondrial membrane (Rossetti et al., 2016a), although other components such as the translocator protein and voltage-dependent anion channel are also involved (Midzak and Papadopoulos, 2016). In the neuronal endoplasmic reticulum, pregnenolone can be converted to progesterone by the 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD). Allopregnanolone production is controlled by the rate-limiting reduction of progesterone into 5α-dyhydroprogesterone by steroid 5α-reductase (5αR), which is then converted by 3α-hydroxysteroid dehydrogenase (3α-HSD) into allopregnanolone (Rossetti et al., 2016a).

Little is known about how the transcriptional control of the mentioned steroidogenic enzymes is differentially conditioned by sex and age in the hippocampus of rodents (Fig. 1). In males, the expression levels of P450scc, 3β-HSD and 3α-HSD have been found to be lower in adult rats than in neonatal ones (Higo et al., 2009; Kimoto et al., 2010), whereas those of StAR and 5αR-1 show no changes. In females, the levels of P450scc, 3β-HSD, 3α-HSD, 5αR-1 and StAR have been recently reported to be lower in middle-aged rats than in young ones (Rossetti et al., 2015). Regarding the expression levels of 5αR-1, 5αR-2 and StAR in the hippocampus, Hojo et al. (2014) and Furukawa et al. (1998) have shown no differences between young adult female and male rats, whereas Kim et al. (2002) showed higher gene levels of StAR in male than in female rats during postnatal weeks 3–10. So far, the molecular mechanisms involved in the transcriptional regulation of these genes are not clear. However, we have recently established a correlation between a decrease in the mRNA expression of hippocampal steroidogenic enzymes and an increase in the methylation status of their promoters in female rats (Rossetti et al., 2015, 2016b).

DNA methylation is defined as the post-synthetic addition of methyl groups to the 5-position of cytosine in a CpG (Cytosine – phosphate – Guanine) site within a DNA strand. A CpG island is defined as a DNA sequence generally greater than 200 bp that significantly deviates from the average genomic pattern by being CpG-rich. Most, or perhaps all, CpG islands, including thousands that are remote from currently annotated promoters, are sites of transcription initiation (Deaton and Bird, 2011). In general, unmethylated regulatory CpG islands are located in tissue-specific expressed genes and in essential housekeeping genes. Methylation of cytosines in such CpG islands leads to the binding of methylated CpG-binding domain proteins, transcription repressors and/or histone deacetylases, thereby blocking gene transcription (Missaghian et al., 2009). DNA methylation is one of the most studied mechanisms for silencing gene expression (Rodenhiser and Mann, 2006).

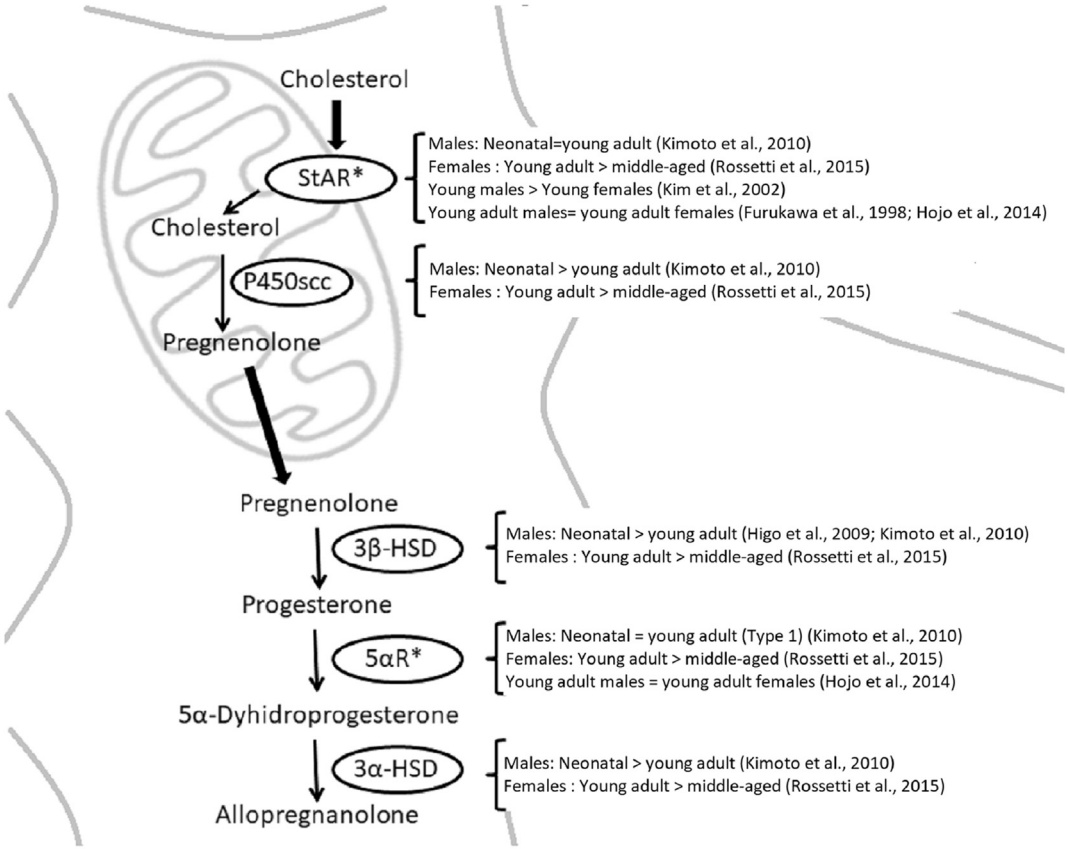

Fig. 1. Pathway of allopregnanolone synthesis in the rat hippocampus and sex/age differences in the gene expression of the enzymes involved. Steroidogenic acute regulatory protein (StAR); cytochrome P450 side chain cleavage (P450scc); 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase(3β-HSD); steroid 5α-reductase (5αR) and 3α-hydroxysteroid dehydrogenase (3α-HSD). *, rate-limiting step.

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The aim of this study was to evaluate the ELM and the transcriptional regulation of hippocampal steroidogenic enzymes in young adult and middle-aged male and female rats. To this end, we evaluated rats in the ELM task and analyzed the mRNA expression and DNA methylation status of the enzymes involved in allopregnanolone synthesis in the rat hippocampus, to elucidate whether epigenetic changes could be involved in their transcriptional regulation. Our findings may contribute to the knowledge of the molecular mechanisms underlying the differences between males and females in brain biology and memory functions during aging.

2. Materials and methods

2.1. Animals and experimental design

Female and male rats of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, 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.

Female (F) and male (M) rats were maintained under standard laboratory conditions and then sacrificed on postnatal day (PND) 90 (young adult; Y) or PND360 (middle-aged; A) during the morning (10 AM) by using CO2 chamber and decapitation (n = 12/group; Fig. 2). The stage of the estrous cycle (proestrus, estrus, metestrus, or diestrus) of each young adult and middle-aged rat was daily determined by vaginal smears for at least 15 days prior to sample collection (Fig. 2); no significant differences in the estrous cycle were observed between the experimental groups. Females were sacrificed during diestrus (Kato et al., 2013). Serum was collected 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.

2.2. ELM task

A three-trial object exploration task in which different versions of the novelty preference paradigm were combined was used to evaluate object recognition memory, memory for locations in which objects were explored, and temporal order memory for objects presented at distinct time points. This protocol was first described by Dere et al. (2005) and Kart-Teke et al. (2006).

2.2.1. Open field

Object exploration was assessed in an open field (80 × 80 × 60 cm) with an open roof. The floor and walls were made of black stainless steel. A video camera, connected to a video recorder, was mounted 100 cm above the field to store the sample and test trials on video tapes for off-line analysis. Diffuse white light provided an illumination density of approximately 3.0 lux at the center of the field. The open roof allowed rats to perceive external distal cues. Proximal visual cues were included on the walls, at a height of 30 cm. After each trial, the apparatus was thoroughly cleaned with a 75% ethanol solution.

2.2.2. Objects A and B

Two different glass objects (in quadruplicate) named as A and B, which differed in terms of height, shape and surface texture, were used. Since the objects were made of the same material, they could not be distinguished by olfactory cues during the test trial. The objects had sufficient weight to ensure that the rats could not displace them or climb them. After each trial, the objects were thoroughly cleaned with a 75% ethanol solution to remove odor cues. Pilot studies ensured that the rats could discriminate between the two objects, and there was no *per se* preference for either object.

2.2.3. ELM task protocol

On PND70 and PND340, female and male rats (n = 12/group) were tested in the ELM task (Fig. 2). First, rats were habituated to the handling procedure, the room and the open field for three

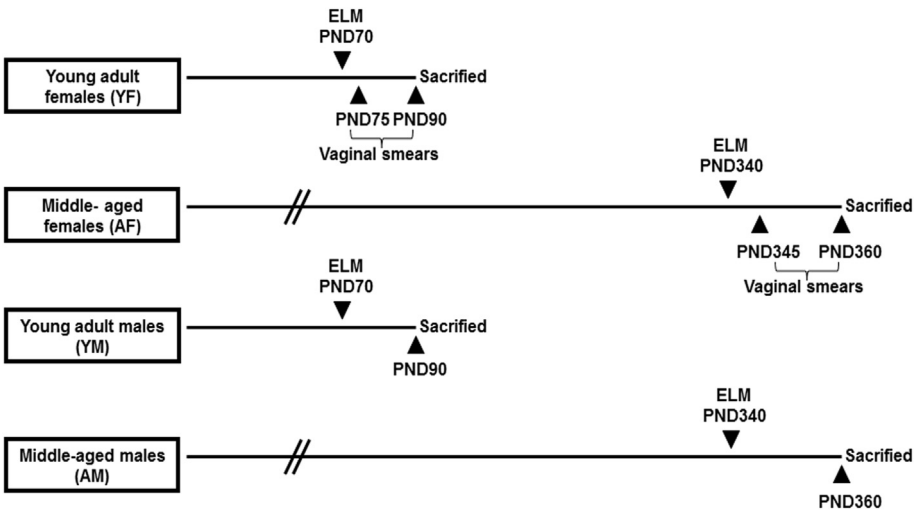

Fig. 2. Experimental design of young adult (Y) and middle-aged (A) female (F) and male (M) rats. Animals were maintained under standard conditions up to post-natal day (PND) 90 and PND360 respectively (n = 12/group). Prior to sample collection, rats were tested in the episodic-like memory (ELM) task and the stage of the estrous cycle of each female rat was daily determined by vaginal smears for at least 15 days.

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consecutive days. To this end, rats received one daily session of 10 min duration. Then, each rat received two sample trials and a test trial (Fig. 3). After placing the rat into the open field, the experimenter left the room to avoid interactions with the animal during testing. On the first sample trial (S1), the rat was placed into the center of the open field, which contained four copies of a novel object (A) arranged in a triangle-shaped spatial configuration, and allowed to explore them for 10 min (Fig. 3). This first object was referred to as the old object. After 50 min, the rat received a second sample trial (S2) identical to the first, except that four novel objects (B) were placed in the open field, in a different spatial configuration, as described in Fig. 3. This second object was referred to as the recent object. After 50 min, the rat received a test trial (T) identical to the second sample trial, except that two copies of the old object and two copies of the recent object were present. One of the old objects and one of the recent objects were kept at a position as presented above (named as Stationary old object and Stationary recent object, respectively) and the others were located in a new position (named as Displaced old object and Displaced recent object, respectively) (Fig. 3).

2.2.4. Variable analysis

The floor of the open field was virtually divided into nine quadrants of equal size and the time spent by each rat exploring the objects was scored off-line from video tapes by using stopwatches. Exploration of an object was assumed when the rat approached an object and had physical contact with it, either with its vibrissae, snout or forepaws.

In addition to the time spent exploring the objects, discrimination ratios named as “when memory” (defined as the proportion of time spent exploring the Displaced recent object against that spent exploring the Stationary recent object) and “where memory” (was defined as the proportion of time spent exploring the Stationary old object against that spent exploring the Stationary recent object) were calculated (Inostroza et al., 2013).

2.3. Hormone assays

Serum levels of estradiol and testosterone were measured in blood samples from each group of rats analyzed (YF, AF, YM and AM) by using Ultra-sensitive Estradiol (DSL 4800) and Testosterone (IM1087) radioimmunoassays (RIAs) (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. The sensitivity of the estradiol assay was 2.2 pg/ml, with intra- and inter-assay coefficients of variation of 8.9% and 12.2%, respectively, whereas the sensitivity of the testosterone assay was 0.03 ng/ml, with intra- and inter-assay coefficients of variation of 5.6% and 15.9% respectively.

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

An optimized qRT-PCR protocol was used to analyze the relative expression levels of steroidogenic transcripts. The hippocampi of six rats 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). Twenty units of ribonuclease inhibitor (RNAout) (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate 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). The primer pairs used for the amplification of StAR, P450scc, 3β-HSD, 5αR-1, 3α-HSD and the ribosomal protein L19 (housekeeping gene) are described in Rossetti et al. (2015). For cDNA amplification, 5 μl of cDNA was combined with HOT FIRE Pol Eva Green 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 yielded no consistent amplification. The relative expression levels of each target gene 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 the qRT-PCR reactions for each target gene 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 gene by the relative standard curve methods, which are designed to analyze data from qRT-PCR (Cikos et al., 2007). For all experimental samples, the relative quantity of each target gene 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 the C_T values were observed for L19 between the various experimental groups.

2.5. Methylation-sensitive analysis

The methylation status of the P450scc, 3α-HSD and 5αR-1

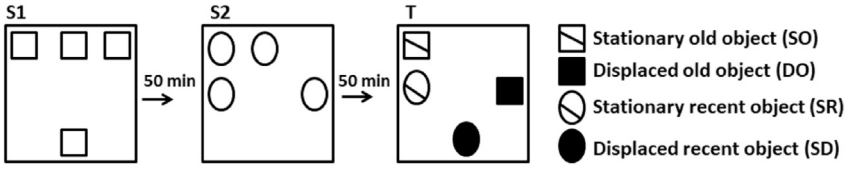

Fig. 3. Episodic-like memory task. Two sample phases (S1 and S2) and a test phase (T) are presented. In S1 and S2, rats explore two sets of four identical novel objects (old objects, S1; recent objects, S2). In the test phase, two objects (one from S1 and the other from S2) are placed in the same location as in the sample phases (Stationary old object and Stationary recent object, respectively). The other two objects are placed in new locations (Displaced old object and Displaced recent object, respectively). Adapted from Inostroza et al. (2013).

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promoters in the experimental groups was evaluated by using a combination of digestion with methylation-sensitive restriction enzymes and subsequent qRT-PCR analysis (Bruce et al., 2008; von Kanel et al., 2010). Hippocampal DNA from each group (n = 6/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 two units of *BstUI* (New England BioLabs, Beverly, MA, USA) or *Mae II* (Roche Applied Science, IN, USA) and 1X enzyme buffer at 60 °C or 50 °C, respectively, in a covered water bath (TecnoDalvo, 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 qRT-PCR protocol was used to analyze the relative methylation levels of various regions of the P450scc, 3α-HSD and 5αR-1 promoters, which have been previously described in Rossetti et al. (2015). The bioinformatics analysis revealed two sites for different methylation-sensitive restriction enzymes in the P450scc promoter studied (named as *Mae II* (a) and *Mae II* (b)), four in the 5αR-1 promoter (*BstUI* (a), *Mae II* (b), *Mae II* (c) and *Mae II* (d)), and three in the 3α-HSD promoter (*Mae II* (a), *Mae II* (b) and *Mae II* (c)) (Rossetti et al., 2015). The StAR and 3β-HSD promoters were also analyzed using bioinformatic tools, but no methylation-sensitive restriction enzymes sites were found. For DNA amplification, 5 μl of DNA was combined with HOT FIRE Pol Eva Green 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. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. The C_T for each sample and the qRT-PCR reaction efficiencies were calculated as described in section 2.3. A region devoid of *BstUI* or *Mae II* restriction sites was amplified as an internal control. When a CpG-rich site is methylated, enzymatic digestion with *BstUI* or *Mae II* is not possible, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *BstUI* 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), which yielded standard curves for the quantification of unknown samples (Cikos et al., 2007).

2.6. Statistical analysis

An exploratory analysis was first conducted 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 using the GraphPad Prism Version 5.03 statistical software package (GraphPad, San Diego, CA, USA). Aging and sex effects on hormone levels, mRNA levels and DNA methylation were analyzed by two-way ANOVA followed by Bonferroni post-test. Data from the ELM task were analyzed by a univariate ANOVA (discrimination ratios or exploratory times) as the within-subject factors and by a multivariate ANOVA (YF, AF, YM, AM) as the between-subject factor. Bonferroni post-test was run to check for differences between groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. The performance in the ELM task is affected by aging in male rats

To analyze whether ELM functions in rats are related to age and sex, we tested them in the "what-where-when" memory triad by using a single-trial object recognition task. During the task, animals encountered two groups of objects (A and B) at different times and locations. One-way ANOVA analyses revealed a significant difference in the exploratory time for each of the four objects in the YM, YF and AF groups (YM, $p = 0.007$; YF, $p = 0.009$; AF, $p = 0.04$; Fig. 5). Therefore, a Bonferroni post-test was done. YM animals exhibited biased exploration, spending more time exploring the Stationary old object than the Stationary recent object ($p < 0.05$; Fig. 5), confirming that they recognized the objects explored during separate trials and remembered their order of presentation. In addition, YM rats also showed differential exploration of displaced objects, spending more time exploring the Displaced recent object than the Stationary recent object ($p < 0.05$; Fig. 5). Overall, these animals preferred to explore mostly the Stationary old object and the Displaced recent object, as previously described by Kart-Teke et al. (2006) in rats and by Pause et al. (2013) in human control subjects. In contrast, AM rats exhibited no clear preference during object exploration ($p > 0.05$; Fig. 5). On the other hand, YF rats preferred to spend more time with the Stationary old object than with the Stationary recent, Displaced old and Displaced recent objects ($p < 0.05$; Fig. 5), whereas AF rats preferred to spend more time with the Stationary old object than with the Stationary recent and Displaced recent objects ($p < 0.05$; Fig. 5). The exploratory time of each group during habituation, sample phases and test phase was measured, but no differences were found between the groups ($p > 0.05$).

As these results showed a different performance in the ELM task during aging in male rats, but not in females, we next examined the existence of different exploration patterns between groups by analyzing the exploratory times for the objects of all experimental groups together. Multivariate ANOVA considering the exploration time for each of the four objects revealed a significant effect of aging ($F_{(3, 189)} = 3.433$; $p = 0.016$; Wilks Lambda = 0.749), not sex ($F_{(3, 189)} = 1.057$; $p = 0.390$; Wilks Lambda = 0.906) and nor interaction effects (age x sex; $F_{(3, 189)} = 0.812$; $p = 0.525$; Wilks Lambda = 0.927). The differences in object exploration between the YM and AM groups were concentrated on the Stationary old object ($p < 0.05$), whereas no changes in the exploratory time per object were found between YF and AF rats. The "where" (spatial component) and "when" (temporal component) ratios were not significantly different between the YF, AF, YM and AM groups (Table 1).

3.2. Estradiol and testosterone serum levels show physiological changes in female and male rats

Since hormone circulating levels could differentially affect ELM and steroidogenic enzyme expression, estradiol and testosterone were measured in serum samples from young-adult and middle-aged female and male rats. Two-way ANOVA analyses revealed the expected physiological sex- and age-related changes in their levels (Fig. 4). Estradiol levels were affected by sex ($F_{(3,28)}$: 7672, $p < 0.0001$), and, regardless of the age, were higher in female rats than in male rats (YF: 18.5 pg/ml ± 3.1; AF: 19.3 pg/ml ± 3.9; YM and AM: below the detection limit of the assay). On the other hand, testosterone levels were conditioned by the interactions between sex and age ($F_{(3,28)}$: 852, $p < 0.005$). The levels of this hormone were significantly different between YM and AM rats (YM: 4.8 ng/ml ± 0.8 ng/ml; AM: 1.25 ng/ml ± 0.15; $p < 0.05$), but undetectable in the serum samples from female rats.

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Table 1
Discrimination ratios corresponding to "when memory" and "where memory" of young adult and middle-aged female (YF and AF) and male (YM and AM) rats during episodic-like memory test.

	YF	AF	YM	AM	P
When Memory	0.42 ± 0.13	0.35 ± 0.11	0.42 ± 0.11	0.47 ± 0.16	P > 0.05
Where Memory - SO	−0.36 ± 0.14	0.04 ± 0.17	−0.32 ± 0.13	−0.13 ± 0.13	P > 0.05
Where Memory - SR	0.05 ± 0.17	−0.11 ± 0.17	0.05 ± 0.17	0.09 ± 0.19	P > 0.05

SO: Stationary old object; SR: Stationary recent object.
A value of zero indicates no preference (chance level), whereas a positive value indicates a preferential exploration of the displaced object for where and of the old stationary object for when.
Results are represent as the means ± SEM (n = 12/group).

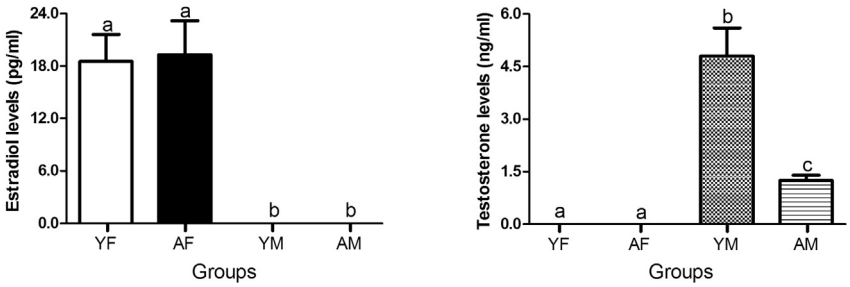

Fig. 4. Serum hormone levels of young adult and middle-aged female (YF and AF) and male (YM and AM) rats. The columns and error bars represent the means ± SEM (n = 12/group). Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after two-way ANOVA.

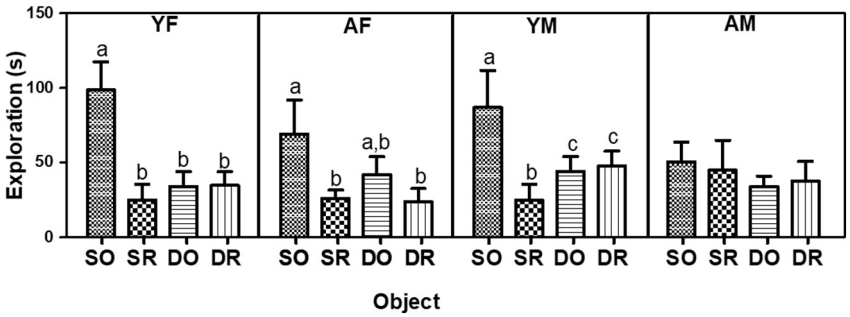

Fig. 5. Performance of young adult and middle-aged female (YF and AF) and male (YM and AM) rats in the episodic-like memory task. Distribution of exploratory times per object: Stationary old object (SO), Stationary recent object (SR), Displaced old object (DO) and Displaced recent object (DR). The columns and error bars represent the means ± SEM (n = 12/group). Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after one-way ANOVA.

3.3. Sex and aging affect the mRNA levels of hippocampal steroidogenic-related genes in rats

To determine whether the transcriptional regulation of the enzymes involved in allopregnanolone synthesis in the hippocampus is age- and/or sex-related, we studied their mRNA expression in young adult and middle-aged female and male rats by using qRT-PCR (Fig. 6). The two-way ANOVA revealed that the expressions of StAR ($F_{(3,28)}$: 37.37, $p < 0.0001$), P450scc ($F_{(3,28)}$: 30.5; $p < 0.0001$), 3β-HSD ($F_{(3,28)}$: 31.87; $p < 0.0001$), 3α-HSD ($F_{(3,28)}$: 26.8; $p < 0.0001$) and 5αR-1 ($F_{(3,28)}$: 28.8; $p < 0.0001$) were affected by the interactions between age and sex. AF rats showed a decrease in the transcription of StAR, P450scc, 3β-HSD, 3α-HSD and 5αR-1, of at least two-fold compared to YF ($p < 0.0001$). This was not observed in male rats (YM vs. AM: $p > 0.05$). In addition, the gene expression of StAR, P450scc, 3β-HSD, 3α-HSD and 5αR-1 was higher in YF than in YM rats ($p < 0.0001$). Middle-aged rats showed a different expression pattern, as only StAR was increased in AM versus AF rats ($p < 0.0001$).

3.4. Sex and aging modify DNA methylation patterns of P450scc, 5αR-1 and 3α-HSD in rats

To determine whether the changes observed in the P450scc, 5αR-1 and 3α-HSD transcript levels were related to DNA methylation modifications, we determined the methylation status of the transcriptionally active promoters of these enzymes in the YF, AF, YM and AM 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. The different sites studied within each promoter (Fig. 7) are referred to as the name of the methylation-sensitive restriction enzymes (*Mae II* or *BstUI*). In the P450scc promoter, the two-way ANOVA revealed that the DNA methylation levels of the *MaeII*(a) site were affected by the interactions between age and sex ($F_{(3,28)}$:14.95, $p < 0.01$). An increase in the methylation status was detected at this site in AF rats compared to YF, YM and AM rats (AF: 1 ± 0.1; YF: 0.6 ± 0.12; YM: 0.56 ± 0.1; AM: 0.55 ± 0.2; $p < 0.01$, Fig. 7A). In the 5αR-1 promoter, the methylation status at the *BstUI* (a), *Mae II* (b) and *Mae*

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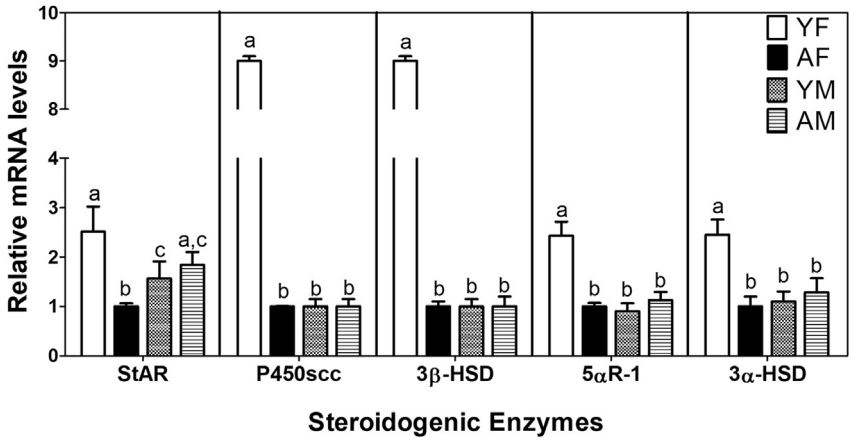

Fig. 6. Real-time quantitative PCR analysis of the mRNA levels of StAR and steroidogenic enzymes in the hippocampus of young adult and middle-aged female (YF and AF) and male (YM and AM) rats housed under standard laboratory conditions. The amounts of mRNA in the YF, YM and AM groups are presented as relative values versus those of the AF group. The columns and error bars represent the means ± SEM (n = 6/group). Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after two-way ANOVA.

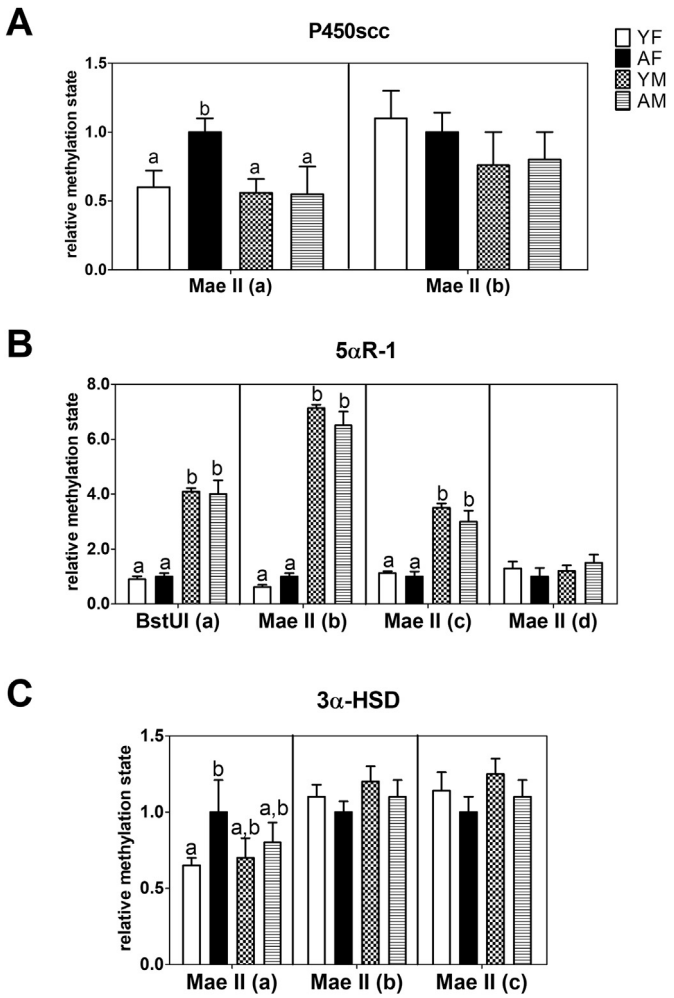

Fig. 7. Methylation analysis using methylation-sensitive restriction enzymes followed by real-time quantitative PCR in the female and male hippocampus of young adult (YF and YM) and middle-aged (AF and AM) rats housed under standard laboratory conditions. Methylation-sensitive restriction sites of the P450scc (A), 5αR-1(B) and 3α-HSD (C) gene promoters were studied. The relative methylation status in the YF, YM and AM groups are indicated as relative values versus those of the AF group. The columns and error bars represent the means ± SEM (n = 6/group). The name of the methylation-sensitive restriction enzyme (*Mae II* or *BstUI*) in each promoter refers to the different position studied. Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after two-way ANOVA.

II (c) sites was increased in YM and AM compared to YF and AF rats by at least three-fold, showing a clear sex-dependent effect ($F_{(3,28)}$ *BstUI* (a): 98.94; $F_{(3,28)}$ *Mae II* (b): 99.38; $F_{(3,28)}$ *Mae II* (c): 97.7; $p < 0.001$, Fig. 7B). In the 3α-HSD promoter, an interaction between the factors studied was found at the *MaeII* (a) site ($F_{(3,28)}$: 23.25; $p < 0.01$) and aging increased the methylation status at this site in female rats (YF: 0.65 ± 0.10; AF:1 ± 0.2, $p < 0.01$, Fig. 7C).

4. Discussion

In the present study, we described the ELM in young adult and middle-aged male and female rats and analyzed the mRNA expression and DNA methylation status of the enzymes involved in allopregnanolone synthesis in the rat hippocampus, in order to elucidate whether epigenetic changes could be involved in their transcriptional regulation. To our knowledge, this is the first study investigating the individual and combined effects of sex and age on this kind of hippocampal memory and on the expression of genes involved in allopregnanolone synthesis in rodents.

4.1. Effect of age and sex on hippocampal-dependent ELM in rats

Aging in rodents is accompanied by changes in specific cognitive functions (Pause et al., 2013; Wallace et al., 2007); however, no studies have analyzed the effects of age on the ELM task in rats. According to our results, in female rats, aging did not affect the performance associated with the temporal component (discrimination between recent and old objects); nevertheless, females were unable to discriminate between displaced and stationary objects at both ages analyzed (PND70 and PND340). Contrary to our results, Diniz et al. (2010) found no differences in the exploratory time per object in 6- and 20-week-old female mice. Regarding male rats, middle-aged ones showed a poorer performance in the ELM task than young adults, suggesting that this kind of memory is impaired in male rats during aging. Similarly, studies using the Morris water maze and the object recognition test, respectively, have shown that aging causes deterioration in the spatial and temporal memory in male rats (Bennett et al., 2006; Wiescholleck et al., 2014). Although these tests are different from those presented here, they support the idea that aging generates significant cognitive impairment associated with spatial and temporal hippocampal-dependent memory, and that this could affect the integration of both kinds of memories. Our results also showed that ELM was sex-related: while males had a good performance in the test on PND70,

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females had difficulty with the spatial component. Besides, while middle-aged female rats were able to discriminate the temporal order in which the objects were presented, males were not. These results are consistent with a work previously published by Sutcliffe et al. (2007), who observed that females showed better performance in the object recognition test and that males were better in the spatial memory test. All these data support the idea that female and male animals are different at hippocampal cognitive levels, and that, particularly, the ELM functions would be conditioned by sex and/or age.

4.2. Effect of age and sex on the transcriptional regulation of the enzymes involved in hippocampal allopregnanolone synthesis in rats: mRNA expression and DNA methylation analysis

In addition to sex variations in behavior and cognition, several works have reported sexual dimorphisms at neurobiological levels such as differences in neurochemical and neuroanatomical properties and gene expression (McCarthy and Arnold, 2011; Ngun et al., 2011; Seeman, 1997; Simpson and Kelly, 2012). Historically, these differences were attributed, although not exclusively, to differences in the secretion of gonadal hormones. However, some authors have highlighted that differences in brain hormone levels may also be related (Ngun et al., 2011; Roof and Hall, 2000).

Here, we found that age and sex have differential effects on the transcription of steroidogenic enzymes. In young adult animals, mRNA expression of StAR, P450scc, 3α-HSD, 3β-HSD and 5αR-1 was significantly higher in the hippocampus of young adult female rats than in that of young adult male rats. In contrast to our findings, Hojo et al. (2014) and Furukawa et al. (1998) found no differences in the expression levels of 5αR-1, 5αR-2 and StAR in the hippocampus of young adult female and male rats. On the other hand, Kim et al. (2002) showed higher gene expression levels of StAR in male than in female rats during postnatal weeks 3–10. Our results are consistent with the higher levels of dihydroprogesterone and allopregnanolone found in the hippocampus of females compared to male rats on PND60 (Caruso et al., 2013). Conversely, the levels of pregnenolone have been found to be higher in male than in female rats on PND60 (Caruso et al., 2013). However, in our study, gene expression of StAR was higher in female than in male rats. The discrepancies between these works could be due to the age of the animals (PND60 vs. PND90) and the fact that the synthesis of steroids is not necessarily reflected in differences in mRNA expression. In fact, it would be interesting to also determine protein levels or enzymatic activity. However, the assessment of these variables has several methodological limitations, such as the low expression of these enzymes in the hippocampus and the lack of specificity of the antibodies available (Higo et al., 2009).

Related to aging, in female rats, the levels of StAR, P450scc, 3β-HSD, 3α-HSD and 5αR-1 mRNA were higher in young adult rats (PND90) than in middle-aged ones (PND360), whereas in males these levels remained low on both PND90 and PND360. Although some authors have described a decrease in hippocampal mRNA expression of P450scc, 3β-HSD and 3α-HSD in male rats with progressive age, these studies have been conducted in neonatal and young adult rats (Higo et al., 2009; Ibanez et al., 2003; Kimoto et al., 2010) and not in middle-aged ones. We are not aware of reports concerning the expression of hippocampal steroidogenic enzymes during neonatal-adult periods in female rats. We have recently reported a decrease in the expression of these enzymes in the hippocampus of PND450 females compared to PND90 ones (Rossetti et al., 2015).

To explain the changes found in the mRNA expression of steroidogenic enzymes in rats, we analyzed the methylation patterns of their promoter regions. We observed hypermethylation at the CpG island of the 5αR-1 promoter in males compared to females. This difference could explain the lower mRNA levels of this enzyme found in young adult male compared to female rats.

We also observed hypermethylation at the P450scc and 3α-HSD promoters in middle-aged compared to young adult female rats, which could explain the decrease in the mRNA expression of these enzymes due to aging. The latter results are similar to those published by our group in adult female rats (PND90 vs. PND450) (Rossetti et al., 2015). Thus, these methylation-sensitive sites could be potential transcriptional regulatory sites. In contrast, the age-dependent changes observed in the transcription of 5αR-1 in females (PND90 vs. PND360) were not reflected in methylation patterns. In addition, no methylation alterations were found in the promoter region of P450scc and 3α-HSD related to the differential expression between young adult male and female rats. These observations could be attributed to the limitations of the technique, since some methylation-targeted CpG sites were not included in the analysis. It is also possible that the transcription of these genes is regulated by other mechanisms such as histone modifications (Martinez-Arguelles and Papadopoulos, 2010) or by activation/inactivation of transcription factors (Christenson and Strauss, 2001; Lin and Penning, 1995; Sher et al., 2007).

4.3. Circulating steroid hormones and their association to brain functions

The hippocampus is a center of learning and memory processes, with a pivotal role in learning about spatial relationships and in the formation of short- and long-term complex associations, e.g., spatial learning and memory in rodents (Escudero et al., 2012). Besides, the hippocampus is known to be a target for neuromodulatory actions of sex hormones, such as estradiol (females) and testosterone (males). In our animals, sex hormone levels were similar to those published previously by other authors (Steger and Peluso, 1982; Zirkin et al., 1993): female rats showed higher levels of estradiol than males, whereas male rats presented increased levels of testosterone than females. In addition, while females maintained similar estradiol levels between young adult and middle-aged periods, males showed an important decline in testosterone levels. These differences are physiological and depend on sex and age. The effects of estradiol on hippocampal memory in rodents have been most commonly assessed in spatial tasks (e.g., Morris water maze, radial arm maze, object location/placement) and in object recognition tasks. Most studies report that systemic or intracranial estradiol administration enhances memory acquisition and consolidation in spatial and object recognition tasks. However, some studies have found no such beneficial effects (Choleris et al., 2012; Frick, 2015; Luine, 2014). On the other hand, positive associations have been found between testosterone levels and global cognition, episodic memory, executive functions and spatial performance in observational studies (Hogervorst, 2013; Hua et al., 2016; Panizzon et al., 2014; Warren et al., 2008; Yeap, 2014). Interestingly, in middle-aged animals, the lower levels of estradiol and testosterone found in male rats could be correlated with the poor performance observed in object recognition (temporal component). Along the same line, the decrease in testosterone levels and the decline in the ELM performance found during aging in male rats could be related. However, more studies are necessary to clarify whether there is a causal relation between estradiol/testosterone levels and ELM functions. Steroid hormones are also correlated to the regulation of neurosteroidogenesis (Bixo et al., 1997; Frye et al., 2011; 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, and increases progesterone levels in the rat

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hypothalamus (Soma et al., 2005). The higher plasma levels of estradiol found in young adult female compared to male rats could explain, at least in part, the higher expression of steroidogenic enzymes described in the former ones.

4.4. Allopregnanolone and memory

We observed lower mRNA expression levels of steroidogenic enzymes in young adult and middle-aged males and in middle-aged female animals than in young adult females. We propose that these results could be related to the presence of lower levels of the neurosteroids associated to these enzymes. Although changes in mRNA expression do not always reflect changes in protein abundance, as we mentioned before, the correlation between mRNA and protein levels of steroidogenic enzymes has previously described. In fact, Dong et al. (2001) correlated an approximately two-fold decrease in the levels of 5αR protein and mRNA with a 50% decrease in allopregnanolone levels in the frontal cortex of socially isolated mice compared to group-housed mice. In addition, the administration of 5αR inhibitors, such as (17β)17[[bis(1-methylethyl) amino]carbonyl] androstane-3,5-diene-3-carboxylic acid and Finasterine, has been reported to decrease the levels of allopregnanolone in the male rat brain (Cheney et al., 1995; Mukai et al., 2008). Several works have reported that progestins (allopregnanolone and progesterone) prevent memory impairment and enhance cognitive performance (Barros et al., 2015; Djebaili et al., 2004; George et al., 2010; Rabinowitz et al., 2014; Singh et al., 2012; Wang et al., 2010) and that the administration of estrogens and/or progestins enhances performance in object recognition tasks but not in placement, water maze, and contextual and cued conditioned fear tasks in rodents (Frye and Walf, 2008; Orr et al., 2009; Walf et al., 2006). On the other hand, allopregnanolone administration alters spatial memory in the Morris Water task in rats (Chin et al., 2011; Johansson et al., 2002; Matthews et al., 2002; Silvers et al., 2003). Similarly, it has been demonstrated that the administration of allopregnanolone impairs episodic memory in healthy women (Kask et al., 2008). Our results in young adult rats are in agreement with these studies: in females, the high expression of the enzymes associated with allopregnanolone synthesis was concomitant with a better performance in object recognition and impairment in ELM, whereas in males, the low expression of these enzymes was concomitant with a good performance in spatial memory and ELM task.

Despite these results, we are not able to establish a cause-effect relationship, since other experiments would be necessary. However, the evidence obtained is novel and has not been described so far and can serve as a basis for future research.

5. Conclusions

The results of the present study show that ELM and the transcriptional regulation of hippocampal steroidogenic enzymes are related to sex and age in rats. We also showed that epigenetic changes are involved in the transcriptional regulation of the enzymes involved in allopregnanolone synthesis in the rat hippocampus. Other experiments would be needed to know whether there is a correlation between ELM and the transcriptional regulation of steroidogenic enzymes in the hippocampus. The results of the present study contribute to the knowledge of sex differences in the transcriptional control of hippocampal steroidogenic genes and memory during aging and may be useful as a basis for future research.

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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