

Accepted Manuscript

The impact of sensory and motor enrichment on the epigenetic control of steroidogenic-related genes in rat hippocampus

Maria Florencia Rossetti, Rocio Schumacher, Gisela P. Lazzarino, Ayelen L. Gomez, Jorgelina Varayoud, Jorge Guillermo Ramos



PII: S0303-7207(19)30037-1

DOI: <https://doi.org/10.1016/j.mce.2019.01.025>

Reference: MCE 10387

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 25 October 2018

Revised Date: 15 January 2019

Accepted Date: 29 January 2019

Please cite this article as: Rossetti, M.F., Schumacher, R., Lazzarino, G.P., Gomez, A.L., Varayoud, J., Ramos, J.G., The impact of sensory and motor enrichment on the epigenetic control of steroidogenic-related genes in rat hippocampus, *Molecular and Cellular Endocrinology* (2019), doi: <https://doi.org/10.1016/j.mce.2019.01.025>.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The impact of sensory and motor enrichment on the epigenetic control of steroidogenic-related genes in rat hippocampus.

Rossetti, Maria Florencia^{a,b}; Schumacher, Rocio^b; Lazzarino, Gisela P.^b; Gomez, Ayelen L.^{b,d}; Varayoud, Jorgelina^{b,c}; Ramos, Jorge Guillermo^{a,b*}.

^a *Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina*

^b *Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral-CONICET, Santa Fe, Argentina.*

^c *Cátedra de Fisiología Humana, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina.*

^d *Cátedra de Patología Humana, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina.*

Email addresses:

mfrossetti@fbc.unl.edu.ar

rociosch09@gmail.com

gplazzarino@fbc.unl.edu.ar

ayelenlucgomez@gmail.com

varayoud@fbc.unl.edu.ar

gramos@fbc.unl.edu.ar

*Corresponding author: Address all correspondence and requests for reprints to Jorge Guillermo Ramos, PhD. Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina. Casilla de Correo 242, (3000) Santa Fe, Argentina. TEL/FAX: 54 342 4510283. E-mail: gramos@fbc.unl.edu.ar

Highlights:

- Sensory and motor enrichment enhance neurosteroidogenic enzyme transcription.
- The effect of enrichment on steroidogenesis differs in young and middle-aged rats.
- The expression of 3α -HSD, 5α R-1 and P450(11 β)-2 is regulated by methylation.

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.

Funding

This work was supported by grants from the Universidad Nacional del Litoral (CAI+D 2011 No 50120110100423 LT) and the Argentine National Agency of Scientific and Technological Promotion (ANPCyT) (PICT 2012 No 1715). These funding sources had no involvement in study design; collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

44

45 *Abstract*

46 In the present study, we analyzed the effects of a short-term environmental enrichment on
47 the mRNA expression and DNA methylation of steroidogenic enzymes in the
48 hippocampus. Thus, young adult (80-day-old) and middle-aged (350-day-old) Wistar
49 female rats were exposed to sensory (SE) or motor (ME) enrichment during 10 days and
50 compared to animals housed under standard conditions. SE was provided by an assortment
51 of objects that included plastic tubes and toys; for ME, rodent wheels were provided. In
52 young adult animals, SE and ME increased the mRNA expression of cytochrome P450
53 17α -hydroxylase/c $17,20$ -lyase, steroid 5α -reductase type 1 (5α R-1) and 3α -hydroxysteroid
54 dehydrogenase and decreased the methylation levels of 5α R-1 gene. In middle-aged rats,
55 ME and SE upregulated the gene expression of aldosterone synthase and decreased the
56 methylation state of its promoter. These results propose that SE and ME differentially
57 regulate the transcription of neurosteroidogenic enzymes through epigenetic mechanisms in
58 young and aged rats.

59

60 *Keywords* (5): sensory enrichment, motor enrichment, steroidogenic enzymes, DNA
61 methylation, hippocampus.

62

63 *Abbreviations*

64 17β -HSD-3: 17β -hydroxysteroid dehydrogenase type 3

65 3α -HSD: 3α -hydroxysteroid dehydrogenase

66 3 β -HSD: 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4-isomerase

67 5 α R-1: steroid 5 α -reductase type 1

68 AD: Alzheimer's disease

69 BDNF: brain-derived neurotrophic factor

70 DG: dentate gyrus

71 DHEA: dehydroepiandrosterone

72 EE: environmental enrichment

73 ME: motor enrichment

74 P450(11 β)-2: aldosterone synthase

75 P450(17 α): cytochrome P450 17 α -hydroxylase/c17,20-lyase

76 P450arom: cytochrome P450arom

77 P450scc: cytochrome P450 side chain cleavage

78 PD: Parkinson's disease

79 SE: sensory enrichment

80 StAR: steroidogenic acute regulatory protein

81 1. Introduction

82 Environmental enrichment (EE) has been defined as “a combination of complex inanimate
83 and social stimulation”(Rosenzweig et al., 1978). Typically, EE is composed of a group of

animals (social stimulation) that are housed in large cages containing different objects of various shapes, sizes and colors (sensory stimulation) and/or running wheels (motor stimulation), that are periodically changed to stimulate curiosity and exploration. In rodents, these experimental conditions are known to improve plasticity (Nithianantharajah and Hannan, 2006; Sale et al., 2009; van Praag et al., 2000), neurogenesis (Beauquis et al., 2010; Valero et al., 2011), synaptogenesis (Birch et al., 2013) and dendritic branching (Beauquis et al., 2010; Bindu et al., 2007). Changes in the expression of neurotrophic and synaptic genes, such as brain-derived neurotrophic factor (BDNF), nerve growth factor and synaptophysin, have also been described (Birch et al., 2013; Nithianantharajah and Hannan, 2006). In addition, several authors have reported that EE enhances memory and learning functions and promotes neuronal protection mechanisms during aging (Birch and Kelly, 2018; Frick and Fernandez, 2003; Leal-Galicia et al., 2008; Mora et al., 2007) and neurodegenerative pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD) and brain injuries (Frick and Benoit, 2010; Laviola et al., 2008; Nithianantharajah and Hannan, 2006; van Praag et al., 2000).

Neurosteroids are steroid hormones synthesized de novo from cholesterol or steroidal precursors in various brain regions, including hippocampus (Compagnone and Mellon, 2000; Reddy, 2010) (Figure 1). They exert several biological actions associated with the central nervous system as a result of genomic actions, mediated by classical receptors (such as progesterone and estrogen receptors), and nongenomic actions, mediated by neurotransmitter receptors (such as GABA and N-methyl-d-aspartate receptors). Neurosteroids regulate critical brain functions including synaptogenesis (Hojo and Kawato, 2018), synaptic plasticity (Reddy, 2010; Rune and Frotscher, 2005), myelination (Brinton, 2013; Mellon, 2007; Reddy, 2010), neuronal survival (Charalampopoulos et al., 2008;

Mellon, 2007; Singh et al., 2012) dendritic growth (Hojo and Kawato, 2018) and cognition (Frye, 2009; Luine, 2014; Schumacher et al., 2003). Moreover, absence or reduced levels of neurosteroids have been reported during aging, neurodegenerative diseases and brain damage (Charalampopoulos et al., 2006; Charalampopoulos et al., 2008; Luchetti et al., 2011a; Luchetti et al., 2011b). Under those conditions, the expression of steroidogenic enzymes has been also found to be affected. For example, damage of cerebellar afferent neurons of the inferior olivary nucleus evoked a significant increase in steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), and cytochrome P450 aromatase (P450arom) mRNA levels at this site (Lavaque et al., 2006). In addition, expression changes were seen for cytochrome P450 17 α -hydroxylase/c17,20-lyase (P450(17 α)) in the rat hippocampus after traumatic brain injury (Birnie et al., 2013). Neurons that expressed P450scc, 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4-isomerase (3 β -HSD), as well as those that expressed 3 α -hydroxysteroid dehydrogenase (3 α -HSD) and steroid 5 α -reductase (5 α R) were lost in adult Niemann Pick Type-C brains, resulting in diminished concentrations of allopregnanolone (Mellon et al., 2008). Alteration levels of 5 α R and P450arom were also found in different brain areas during PD, AD and/or multiple sclerosis (Luchetti et al., 2011a; Luchetti et al., 2011b). All these findings strongly suggest a key role of neurosteroids in neuroprotection.

Little is known about the association between environment and brain steroidogenesis. In hippocampus, social isolation has been shown to decrease the mRNA levels of the 5 α R type 1 (5 α R-1) in mice (Agis-Balboa et al., 2007) and increased the level of P450arom mRNA and stimulated estradiol synthesis in the rats (Munetsuna et al., 2009). Contrary, Munetsuna et al. (2011) reported an increase in the levels of mRNAs for 5 α R-1 and 3 α -HSD in the hippocampus of young adult male rats exposed to a long-term EE. Recently, we found that

this experimental condition also increased the expression of 5 α R-1, 3 α -HSD and P450(17 α) in the hippocampus of middle-aged female rats (Rossetti et al., 2015). Moreover, we related these changes in the expression of 5 α R-1 with alterations in the methylation levels of its promoter. DNA methylation involves the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine and is one of the most studied mechanisms for silencing gene expression (Moore et al., 2013). In fact, DNA methylation mechanism has been shown to regulate the transcription of steroidogenic enzymes such as P450arom (Lazzarino et al., 2017; Vanselow et al., 2005; Vanselow et al., 2010), P450(17 α) (Missaghian et al., 2009), 5 α R-1 (Lazzarino et al., 2017; Rossetti et al., 2016b; Rossetti et al., 2015), 3 α -HSD (Rossetti et al., 2015), and aldosterone synthase (P450(11 β)-2) (Howard et al., 2014; Yoshii et al., 2016) suggesting that steroidogenesis is under epigenetic control.

In order to understand how environment improves neuronal functions, several studies have identified a plethora of cellular, molecular and behavioral changes associated with the beneficial effects of EE. However, a comprehensive analysis is necessary to identify the impact of this kind of stimuli on neurosteroidogenesis. Until now, all studies have been conducted using long-term enriched conditions. Here, we analyzed for the first time the effects of a short-term EE on brain steroidogenesis. For that, we measured mRNA expression of steroidogenic enzymes in the hippocampus of young adult and middle-aged female rats exposed during 10 days to a sensory or motor enrichment. In addition, we demonstrated the localization of the most relevant steroidogenic enzymes using immunohistochemistry (IHC). Finally, the methylation levels of the promoter regions of steroidogenic enzymes were analyzed as a possible mechanism of regulation of transcription.

2. Materials and methods

2.1. Animals and Experimental Design

Young adult (Y) and middle-Aged (A) female rats of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) with free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water supplied ad libitum in glass bottles with rubber stoppers surrounded by a steel ring. Rats were conserved under standard laboratory conditions (SC) up to postnatal day (PND) 80 (Y) or PND 350 (A). At these ages, rats were divided into six groups: 1)- animals exposed to sensory enrichment (SE) until PND 90 or 360 (Y-SE and A-SE, respectively); 2)- animals exposed to motor enrichment (ME) until PND 90 or 360 (Y-ME and A-ME, respectively) and 3)- animals left in standard laboratory conditions up to the same ages (Y-SC and A-SC). The time course of the experiment is displayed in Figure 2.

Standard and enriched rats were housed in a group of eight animals in large cages that were designed to provide social stimulation. The first ones were not exposed to enriching objects. Enriched rats were divided into two groups: those exposed to SE and those exposed to ME. SE was provided by an assortment of objects that always included large plastic tubes and toys of various shapes, sizes and colors (Figure 2). For ME, rodent wheels were provided (Figure 2). Same partners were maintained during all the experiment. Enrichment protocols were described previously by our group in Rossetti et al. (2015). Animals were

179 handled humanely and with regard for the alleviation of suffering and in accordance with
180 the principles and procedures outlined in the Guide for the Care and Use of Laboratory
181 Animals issued by the US National Academy of Sciences and approved by the ethical
182 committee of the School of Biochemistry and Biological Sciences, Universidad Nacional
183 del Litoral.

184 Animals were sacrificed by decapitation at PND 90 (Y-SC, Y-SE, Y-ME) or PND 360 (A-
185 SC, A-SE and A-ME). Importantly, all animals were sacrificed during the diestrous phase
186 of the estrous cycle in order to maintain similar endocrine conditions. No significant
187 differences in estrous cycle were observed between young adult and middle-aged rats
188 (Rossetti et al., 2016b). The hippocampus (n=16/group) was quickly microdissected under
189 a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY, USA), frozen in liquid
190 nitrogen and kept at -80°C for mRNA and DNA. For IHC, brains were fixed in 40%
191 buffered paraformaldehyde and embedded in paraffin.

192 2.2. mRNA analysis

193 2.2.1. RNA extraction and reverse transcription

194 The hippocampi of eight animals from each experimental group were individually
195 homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA was prepared according
196 to the manufacturer's protocol. The concentration of total RNA was assessed by A260, and
197 the samples were stored at -80°C . 4 μg of total RNA were reverse-transcribed into cDNA
198 with Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega, Madison,
199 WI, USA) using 200 pmol of random primers (Promega, Madison, WI) as was described by
200 Rossetti et al. (2015).

2.2.2. Real time quantitative PCR (RT-PCR)

Each reverse-transcribed product was amplified in duplicate using the Real-Time DNA Step One Cyclers (Applied Biosystems Inc., Foster City, CA, USA). Primer pairs used for the amplification of StAR, P450scc, 3 β -HSD, P450(17 α), P450arom, 5 α R-1, 3 α -HSD, P450(11 β)-2, 17 β -hydroxysteroid dehydrogenase type 3 (17 β -HSD-3), and the ribosomal protein L19 (housekeeping gene) are shown in Rossetti et al. (2015). For cDNA amplification, 5 μ l of cDNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 μ l. 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 agarose gel electrophoresis. Controls containing no template DNA were included in all assays. The relative expression levels of each target were calculated based on the cycle threshold (C_T) method (Higuchi et al., 1993), which was calculated using the Step One Software (Applied Biosystems Inc. Foster City, CA, USA, RRID: SCR_014281) with an automatic fluorescence threshold (R_n) setting. The efficiency of the PCR reactions for each target was assessed by the amplification of serial dilutions 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 (Rossetti et al., 2015). No significant differences in C_T values were observed for L19 among the various experimental groups.

2.3. Immunohistochemistry

Brain serial sections (5 μ m in thickness) were deparaffinized and dehydrated in graded ethanols. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary antibodies against 5 α R-1 and P450arom were incubated overnight at 4 °C (Table 2). The reactions were developed using a streptavidin-biotin peroxidase method and a combination of diaminobenzidine and nickel chloride (DAB-Ni) (Sigma) or DAB alone was used as chromogen substrate. Samples were mounted with permanent mounting medium (Eukitt, Sigma). Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune goat serum (Sigma).

The images of different hippocampal fields were recorded by a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (illumination, 12 V, 100 W halogen lamp, equipped with a stabilized light source). The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and the calibration of the measurement system were performed with a reference slide. The granule layers of the dentate gyrus (DG), and the CA1 and CA3 regions of the hippocampus were delimited as was previously described by Moreno-Piovanio et al. (2014).

2.4. Generation of anti-5 α R-1 and anti- P450arom and validation assays

For 5 α R-1 and P450arom, we generated affinity-purified rabbit polyclonal antibodies, following previously described protocols (Varayoud et al., 2008). The antigens were expressed in *Escherichia coli* JM109 (Stratagene Corp., La Jolla, CA, USA) as glutathione-S-transferase fusion proteins using a pGEX4T-3 vector (Stratagene Corp., La Jolla, CA). The 5 α R-1 antigen included the region corresponding to amino acids 157 - 209 of the rat

sequence (Accession no. Genbank: NM_017070.3), whereas the P450arom antigen included the region corresponding to amino acids 341 – 400 (Accession no. Genbank: NM_017085.2). Antibodies were purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column; GE Healthcare, Buenos Aires, Argentina). For specificity validation tests, the antibodies against steroidogenic enzymes were preabsorbed by incubating 1 µg of antibody with 10–20 µg of the antigenic peptide for 24 h at 4 °C and both antibody–antigen complexes were assayed by Western blot and IHC in positive control tissue. In addition, the specificity of the anti-serum was tested by Western blot analysis of protein extracts from rat ovary and by IHC of ovary sections.

For Western blot, protein extractions were performed as previously described (Kass et al., 2012). Total protein concentration was determined using a colorimetric BCA Protein Assay Kit from Pierce Chemical Co. (Rockford, IL, USA). Equal amounts of protein (100 µg) from each sample were resolved by 15% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Argentina, Buenos Aires, Argentina) and reacted with anti-5αR-1 (dilution 1:100, 1:200 and 1:400) and anti-P450arom (dilution 1:100, 1:200 and 1:400). A peroxidase-conjugated anti-rabbit secondary antibody (Sigma–Aldrich) was used at a 1:200 dilution, and the reaction was visualized using diaminobenzidine (DAB, Sigma–Aldrich). Molecular weights were determined by comparison to molecular weight standards (Broad Range Protein Markers, Promega, Madison, WI, USA).

2.5. Characterization of anti-5αR-1 and anti- P450arom

The characterization of the antiserum obtained against 5αR-1 and P450arom is shown in Figure 3. Specific bands of 29 kDa and 58 kDa were detected in Western blot assays in the ovary of pregnant rat on gestational day 17, respectively (Figure 3A). To evaluate the

immunoreactivity in paraffin-fixed tissues, IHC assays on ovary sections were performed (Table 2) and the expression pattern of both proteins was defined (Figure 3B). Immunoreactivity of P450arom was observed as a dotted cytoplasmic pattern compatible with a mitochondrial located protein in the cytoplasm of oocytes (Figure 3B-a); staining for 5 α R-1 was found in the cytoplasm of the ovarian granulosa and theca cells (Figure 3B-b). Theca cells also presented lower positive immunoreactivity for P450arom. Specific staining was absent when the antibodies were preincubated with the corresponding peptide used as immunogen, indicating the specificity of the Western blot and IHC staining (Figure 3B-c,d).

2.6. Methylation-sensitive analysis

2.6.1. Bioinformatic analysis

Promoter region of 3 α -HSD (Rossetti et al., 2015), 5 α R-1 (Rossetti et al., 2015), P450arom (Rossetti et al., 2016b) and P450(11 β)-2 (Rossetti et al., 2016b) were previously described by our group. Here, we analyzed the P450(17 α) rat gene (Accession number NC_005100.4) using MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>; RRID: SCR_010269) for CpG islands, which are 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 tested for methylation sensitive restriction *Bst*UI, *Sma*I and *Mae* II enzymes and for putative binding sites for transcription factors using the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>; RRID: SCR_004262). PCR primers were designed using the NCBI Primer-BLAST

(National Center for Biotechnology; <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>;
RRID: SCR_003095) (Table 1).

2.6.2. DNA extraction and digestions with methylation-sensitive restriction enzymes

Hippocampal DNA (n=8/group) was individually prepared using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The concentration of total DNA was assessed by A_{260} , and DNA was stored at 2–8 °C until needed. Equal quantities (1.5 µg) of total DNA were digested with 7.5 units of BamHI (Promega, Madison, WI) to reduce the size of the DNA fragments and then purified with the Wizard SV gel and PCR Clean-Up System Kit (Promega, Madison, WI). A 130 ng sample of BamHI-cleaved DNA was digested overnight with 2 units of *Bst*UI (New England BioLabs, Beverly, MA) or *Mae* II (Roche Applied Science, Indianapolis, IN) and 1X enzyme buffer at 60 °C or 50 °C, respectively, as described by Rossetti et al. (2015).

2.6.3. Real time quantitative PCR (RT-PCR)

An optimized PCR protocol was employed to analyze the relative expression levels of various regions of the 3 α -HSD (Rossetti et al., 2015), 5 α R-1 (Rossetti et al., 2015), P450arom (Rossetti et al., 2016b), P450(11 β)-2 (Rossetti et al., 2016b) and P450 (17 α) (Table 1) promoters. For DNA amplification, 5 µl of DNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 µl. Each sample was quantified in triplicate. Primer pairs and PCR procedures were described previously in Rossetti et al. (2015) and Rossetti et al. (2016b). A region devoid of *Bst*UI or *Mae* II restriction sites was amplified as an internal control (IC). When a CpG-rich site is

314 methylated, enzymatic digestion with *Bst*UI or *Mae* II is not possible, allowing
 315 amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *Bst*UI
 316 or *Mae* II cleaves the DNA and prevents amplification of the fragment. The relative degree
 317 of promoter methylation was calculated by plotting C_T values against the log input (internal
 318 control), yielding standard curves for the quantification of unknown samples (Rossetti et
 319 al., 2015).

321 2.7. Statistical analysis

322 Sample size was determined using G Power software (<http://www.gpower.hhu.de/>;
 323 RRID:SCR_013726) (Faul et al., 2007). To confirm normal distribution and
 324 variance homogeneity an exploratory analysis was conducted (Shapiro–Wilk test, Levene's
 325 test). Data (expressed as the means \pm SEM) were statistically analyzed using the IBM SPSS
 326 Statistics 19 software (IBM Inc.; RRID: SCR_002865). SE and ME effects on mRNA
 327 levels and DNA methylation were analyzed by two-way ANOVA followed by Bonferroni
 328 post-test. Differences were considered significant at $p < 0.05$.

329 3. Results

330 3.1. Sensory and motor enrichment differentially modify the mRNA expression 331 of hippocampal steroidogenic genes

332
 333 Two-way ANOVA revealed interaction between age and environmental stimulus in
 334 the transcription of P450(17 α) ($F_{(3,28)}$: 3.63, $p < 0.05$), 5 α R-1 ($F_{(3,28)}$: 4.11, $p < 0.05$), 3 α -
 335 HSD ($F_{(3,28)}$: 3.71, $p < 0.05$), P450(11 β)-2 ($F_{(3,28)}$: 3.69, $p < 0.05$) and P450arom ($F_{(3,28)}$:

21.68, $p < 0.001$). The expression of P450(17 α), 5 α R-1 and 3 α -HSD decreased in middle-aged rats (A-SC, A-SE and A-ME) compared to the young adult groups (Y-SC, Y-SE and Y-ME), independently of the environment (standard/enrichment, Figure 4). In addition, the mRNA levels of P450(17 α), 5 α R-1 and 3 α -HSD increased in both groups Y-SE and Y-ME compared to Y-SC ($p < 0.05$, Figure 4). On the other hand, gene levels of P450(11 β)-2 and P450arom were higher in the Y-SC compared to the A-SC group ($p < 0.05$), but a different result was found when compare young and aged rats exposed to EE (Figure 4). In young adult animals, ME increased the levels of the P450(11 β)-2 gene (Y-SC vs. Y-ME, $p < 0.05$); in middle-aged animals both SE and ME improved the transcription of this enzyme (A-SC vs. A-ME, $p < 0.05$, A-SC vs. A-SE, $p < 0.05$), achieving mRNA levels in A-ME and A-SE equal to levels in Y-SC group (Figure 4). Contrary, a decreased in the expression of P450arom was detected in Y-ME and Y-SE compared to Y-SC animals ($p < 0.05$, Figure 4). The gene expression of StAR ($F_{(3,28)}: 91$, $p < 0.001$), P450scc ($F_{(3,28)} > 1000$, $p < 0.001$), 3 β -HSD ($F_{(3,28)} > 1000$, $p < 0.001$) and 17 β -HSD-3 ($F_{(3,28)}: 89.9$, $p < 0.001$) decreased by at least 2-fold in middle-aged rats (A-SC, A-SE and A-ME) compared to the young adult group (Y-SC, Y-SE and Y-ME), but no effect of enrichment condition was detected (Figure 4).

3.2. Localization of 5 α R-1 and P450arom proteins in the rat hippocampus

The localization of the most relevant steroidogenic enzymes 5 α R-1 and P450arom was determined in hippocampal formation using IHC (Figure 5). Immunoreaction was restricted to the cytoplasm of neurons and was observed as a speckled-labeling pattern in neurons located in the CA1-CA3 and DG regions. The pattern of expression did not show differences between all experimental groups. Taking into account that the expression of

these proteins was extremely low in both the young adult and middle-aged rat hippocampus, it was not possible to perform a quantitative analysis.

3.3. *In silico* analysis of candidate sites of DNA methylation in the rat P450(17 α) gene promoter

The 3 α -HSD (Rossetti et al., 2015), 5 α R-1(Rossetti et al., 2015), P450arom(Rossetti et al., 2016b) and P450(11 β)-2(Rossetti et al., 2016b) promoter regions, their predicted binding proteins and methylation-targeted CG areas have been previously described. In this study, we analyzed the promoter region of the P450(17 α) looking for methylation-targeted CG areas. Using informatic tools, we found a CpG Island within the promoter region and one site for *Mae II* enzyme. In addition, one 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*. No relevant transcription factors were predicted in the study areas. The results are shown in Figure 6.

3.4. Methylation levels of steroidogenic enzyme promoters are altered by sensory and motor enrichment

To evaluate whether the effects of sensory and motor stimulation on the transcript expression were associated with differential DNA methylation, we determined the methylation state of the transcriptionally active promoters of steroidogenic enzymes in all experimental groups. For that, genomic DNA 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 are referred to as the name of the methylation-sensitive restriction enzymes (*Mae II* or *BstUI*).

Interactions between age and environment were detected in *MaeII*(c) site from 3 α -HSD promoter ($F_{(3,28)} : 6.42, p < 0.01$), *BstUI*(a) ($F_{(3,28)} : 7.17, p < 0.01$) and *MaeII*(d) ($F_{(3,28)} : 6.98, p < 0.01$) sites in 5 α R-1 promoter and at the *MaeII*(a) site in P450(11 β)-2 promoter ($F_{(3,28)} : 4.53, p < 0.05$). Particularly, a decrease at the *MaeII* (c) site was found at the 3 α -HSD promoter in Y-ME, A-SE and A-ME rats compared to Y-SE and Y-SC ($p < 0.05$, Figure 7A). In addition, in the 5 α R-1 gene a reduction in the methylation status was detected at the *BstUI*(a) site in Y-ME rats compared to Y-SE and Y-SC ($p < 0.01$, Figure 7B), while *MaeII*(d) site methylation levels decreased in the Y-SE and A-SE compared to Y-SC, Y-ME and A-ME ($p < 0.01$, Figure 7B). Finally, methylation levels of *MaeII*(a) site in P450(11 β)-2 promoter ($p < 0.05$, Figure 7D) were higher in Y-SC, Y-ME and A-SC compared to Y-SE, A-SE and A-ME rats. No changes were detected in methylation levels of the P450(17 α) and P450arom genes (Figure 7C and 7E).

4. Discussion

Several studies have identified changes at cellular, molecular and behavioral levels associated with the effects of a short-term EE. Here, we analyzed for the first time the effects of this experimental environment condition on the transcriptional regulation of steroidogenic enzymes in the hippocampus of young and middle-aged female rats, differentiating sensory and motor enrichment in two independent experiments. In young animals, both kind of environments SE and ME increased the mRNA expression of

P450(17 α), 5 α R-1 and 3 α -HSD compared to SC group. These results are similar to those found by Munetsuna et al. (2011) and by our group (Rossetti et al., 2015) in adult animals that were exposed to a long-term EE (8 or 16 weeks, respectively). On the other hand, SE and ME decreased the expression of P450arom compared to animals housed under SC. Interestingly, ME upregulated P450(11 β)-2 gene expression in both young adult and middle-aged animals. No other differences in gene expression were observed in aged animals. These results are in accordance with other authors that show the differential effect of EE on memory functions and synaptic plasticity, depending on the age of the animals. While some of them found a positive effect on object recognition and spatial memory in aged animals exposed to a long-term EE (Bennett et al., 2006; Frick et al., 2003; Gresack et al., 2007a, 2007b; Harburger et al., 2007); others reported that a short-term EE only improves brain functions, such as synaptogenesis and neurogenesis, in young animals, but not in aged ones (Rapley et al., 2018; Sager et al., 2018). In addition to that, we previously reported that the exposure of middle-aged rats for 12 weeks to EE increased mRNA levels of 5 α R-1, 3 α -HSD and P450(17 α) (Rossetti et al., 2015). The results suggest that middle-aged animals would require a more prolonged stimulus than do young adults to observe similar effects and this could be a consequence of age-associated cognitive impairment, decreased plasticity (Bishop et al., 2010; Schumacher et al., 2003) and decline of neurosteroidogenesis (Higo et al., 2009; Hojo and Kawato, 2018). In this sense, we found that the mRNA levels of steroidogenic enzymes were 2.5- to 9- fold higher in young adult rats than in middle-aged rats. No differences in estrous cycle and circulating estradiol concentration were reported between these ages (Rossetti et al., 2018; Rossetti et al., 2016b). However, estradiol levels were quantified only during diestrous phase; thus, we could not discard estradiol concentration changes during proestrous/estrous phases. Based

on that and taking into account the relevance of estradiol in the control of neurosteroidogenesis, the variations in ovarian status could be a possible factor that explains the modification in the transcription of steroidogenic enzymes associated with age. Nevertheless, further experiments are needed. Several studies have reported a decrease of steroidogenic enzyme expression during postnatal development (Higo et al., 2009; Ibanez et al., 2003; Kim et al., 2002; Kimoto et al., 2010; Rossetti et al., 2018; Rossetti et al., 2015). Although it would be interesting to evaluate if all these changes at mRNA level correlate with changes in protein expression, a quantitative comparison using Western Blot and/or IHC is very difficult, due to extremely low level expression of these proteins (Higo et al., 2009). In the present study we showed the localization of 5 α R-1 and P450arom enzymes and the results are in accordance with those previously mentioned by Hojo et al. (2011). The pattern of expression of these molecules in CA1-CA3 and DG was similar between all experimental groups.

Epigenetic dysregulation currently garners much attention as a pivotal player in aging and age-related neurodegenerative disorders, where it may mediate interactions between genetic and environmental factors (Delgado-Morales et al., 2017; Gasparoni et al., 2018; Lardenoije et al., 2015; Nativio et al., 2018). In this sense, previous studies reported modifications in epigenetic programming by different types of environments. Weaver et al (2004) reported that increased pup licking and grooming and arched-back nursing by rat mothers altered the offspring epigenome at a glucocorticoid receptor gene promoter in the hippocampus. Kuzumaki et al. (2011) showed that the induction of BDNF expression is correlated with significant changes in histone methylation in the hippocampus of mice exposed to EE. Recently, we showed alterations in methylation levels of steroidogenic enzymes such as 5 α R-1 and P450arom by long-term EE, maternal experience and junk-

food diet exposure (Lazzarino et al., 2017; Rossetti et al., 2016b; Rossetti et al., 2015). Along the same line, we observed hypomethylation at the 5 α R-1 gene (site d) produced by SE and at the 5 α R-1 (site a) and 3 α -HSD promoters produced by ME, in young rats. The fact that two different sites within the CpG Island of the 5 α R-1 promoter altered their methylation patterns depending on the EE, suggests that these sites could be potentially regulatory stimulus-specific sites. These results are in accordance with a recent publication by our group where we correlated a hippocampal alterations in the methylation levels of 5 α R-1 (site d) with changes in the mRNA expression of this enzyme in young adult rats exposed to a long-term EE (Rossetti et al., 2015). In aged rats, SE and ME decreased methylation levels at the P450(11 β)-2. Although this site is not included in a CpG Island, it corresponds with a *cis-acting* element Ad1, which is a regulatory site localized in the 5' upstream promoter region (Nomura et al., 1993). Altogether, these results proposed that a short-term SE and ME could increase the gene expression of 3 α -HSD, 5 α R-1 and P450(11 β)-2 in the hippocampus through methylation changes in their promoters.

Alterations in the methylation patterns at the P450(11 β)-2 promoter were found in young animals exposed to SE, although no mRNA levels changes were reported. In this sense, it is possible that a greater change in methylation control is required to modify mRNA levels. In addition, further experiments are needed to analyze the role of the promoter study site in the transcriptional control of the associated enzyme and to examine the relevance of these epigenetic changes. Contrary, no methylation alterations were found in P450arom and P450(17 α) promoters. Due to the limitations of the technique, some methylation-targeted CG sites were not included in the analysis. In addition, EE could regulate the transcription of these enzymes by other epigenetic mechanisms, such as histone

modifications or miRNA (Martinez-Arguelles and Papadopoulos, 2010; Zhang and Ho, 2011), or by controlling the expression of certain transcription factors (Yang et al., 2002). Slight changes in steroidogenic enzyme expression have been previously shown to affect metabolism. A 2-fold decrease in the levels of 5 α R protein and mRNA was correlated with 50% reduction in allopregnanolone levels in the frontal cortex of mice (Dong et al., 2001). In addition, Higo et al. (2009) showed a correlation between an increase in the rate of metabolism for androgens and estrogen and a rise (1.3- to 1.5-fold) of the mRNA expression of steroidogenic-related enzymes such as P450arom and P450(17 α) in the hippocampus of male rats at PND10. In this context, it is possible that the major changes found in 5 α R-1, 3 α -HSD, P450(17 α), P450arom, and P450(11 β)-2 enzyme expression in the female hippocampus exposed to a short-term EE could be associated with alterations in neurosteroid levels such as allopregnenolone, dehydroepiandrosterone (DHEA), corticosteroid and estradiol.

Short-term cognitive enrichment and/or voluntary exercise has been reported to improve hippocampal neurogenesis (Beauquis et al., 2010; Brenes et al., 2016), dendritic branching (Beauquis et al., 2010; Bindu et al., 2007), neuron survival (Birch et al., 2013), synaptic plasticity (Ashokan et al., 2016; Bhagya et al., 2017; Cassilhas et al., 2016; Ryan and Kelly, 2016; Stein et al., 2016), neurotrophin expression (Birch et al., 2013; Sleiman et al., 2016) and memory performance (Bhagya et al., 2017; Birch et al., 2013; Cassilhas et al., 2016; Diederich et al., 2017; Griffin et al., 2009). Interestingly, Lambert et al. (2005) provided the first evidence that different elements of the EE (cognitive enrichment versus voluntary exercise) have markedly distinct effects on spatial memory and synaptic alterations. Particularly, exercise, but not cognitive stimulation, improved spatial working memory relative to controls, despite the fact that both exercise and cognitive stimulation

increased synaptophysin levels in the neocortex and hippocampus. In this sense, our results show how sensory and motor stimuli differentially modify the transcription of hippocampal steroidogenic enzymes: while both SE and ME enhanced the expression of enzymes involved in allopregnanolone and DHEA synthesis in young adult animals, ME improved the transcription of genes that are implicated in corticosteroid pathway in young adult and middle-aged rats. Allopregnanolone increases hippocampal neurogenesis, neuronal survival and density of dendritic spines, improves learning and memory functions and has a neuroprotective role in diseases such as AD, PD and multiple sclerosis (Rossetti et al., 2016a). On the other hand, corticosteroids are widely known by their anti-inflammatory effects. Remarkably, Du et al. (2017) previously reported that exercise training increases 11β -hydroxysteroid dehydrogenase 1 expression, contributing to glucocorticoid activation and suppression of pulmonary inflammation in obese mice. In addition, several works reported that corticosteroids are involved in memory formation (Lupien and Lepage, 2001) and consolidation in the rodent hippocampus (Cottrell and Nakajima, 1977; Kelemen et al., 2014; Micheau et al., 1984). DHEA and its sulfate form contribute to the survival of neurons and the growth of neurites of the cortical neurons of embryonic rat brains, have a protective effect on hippocampal neurons against the toxic effects of glutamate, and have exhibited anti-oxidant and anti-inflammatory effects (Delchev and Georgieva, 2018). In this context, it is possible that sensory and motor enrichment regulate the transcription of steroidogenic enzymes to improve the levels of certain neurosteroids and promote hippocampal neuronal plasticity and neuroprotection.

The results related to P450arom expression detected in the present study are contradictory with previous works. Surprisingly, both SE and ME decreased the expression of P450arom in young adult rats. Several authors found that P450arom appears to play a pivotal role in

neuroprotection by increasing production of estrogen at the site of mechanical or ischemic injury (Lavaque et al., 2006; Luchetti et al., 2011a; Luchetti et al., 2011b; Roselli, 2007). Thus, we could propose that the decrease expression of P450arom that we found here, could be part of a compensatory mechanism in response to the increase transcription of enzymes involved in other steroid pathways such as P450(17 α), 5 α R-1 (in SE and ME animals) and P450(11 β)-2 (in ME animals). However, further studies are needed to establish the importance of neurosteroids under these experimental conditions.

5. Conclusion

We analyzed the effects of a short-term EE on brain steroidogenesis, differentiating sensory and motor enrichment in two independent experiments. In young adult animals, both kind of environments increased the mRNA expression of P450(17 α), 5 α R-1 and 3 α -HSD enzymes and decreased the expression of P450arom. Moreover, we found hypomethylation at the 5 α R-1 gene produced by both SE and ME at different regulatory sites and at the 3 α -HSD promoters produced by ME. In aged rats, ME upregulated P450(11 β)-2 gene expression and decreased the methylation levels of its promoter. In this context, we proposed that sensory and motor enrichment could differentially regulate the transcription of steroidogenic enzymes through epigenetic mechanisms. Although further studies are needed, these changes could improve the levels of neurosteroids and potentially promoted the plasticity and the neuronal functions.

6. Acknowledgments

We thank Juan Grant and Juan C. Villarreal for technical assistance and animal care and Stella Vaira from the Mathematics' Department (Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina).

7. References

- Agis-Balboa, R. C., Pinna G., Pibiri F., Kadriu B., Costa E., Guidotti A., 2007. Down-regulation of neurosteroid biosynthesis in corticolimbic circuits mediates social isolation-induced behavior in mice. *Proc Natl Acad Sci U S A.* 104, 18736-18741. <http://dx.doi.org/10.1073/pnas.0709419104>
- Ashokan, A., Hegde A., Mitra R., 2016. Short-term environmental enrichment is sufficient to counter stress-induced anxiety and associated structural and molecular plasticity in basolateral amygdala. *Psychoneuroendocrinology.* 69, 189-196. <http://dx.doi.org/10.1016/j.psyneuen.2016.04.009>
- Beauquis, J., Roig P., De Nicola A. F., Saravia F., 2010. Short-term environmental enrichment enhances adult neurogenesis, vascular network and dendritic complexity in the hippocampus of type 1 diabetic mice. *PLoS One.* 5, e13993. <http://dx.doi.org/10.1371/journal.pone.0013993>
- Bennett, J. C., McRae P. A., Levy L. J., Frick K. M., 2006. Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. *Neurobiol Learn Mem.* 85, 139-152. <http://dx.doi.org/10.1016/j.nlm.2005.09.003>
- Bhagya, V. R., Srikumar B. N., Veena J., Shankaranarayana Rao B. S., 2017. Short-term exposure to enriched environment rescues chronic stress-induced impaired hippocampal synaptic plasticity, anxiety, and memory deficits. *J Neurosci Res.* 95, 1602-1610. <http://dx.doi.org/10.1002/jnr.23992>
- Bindu, B., Alladi P. A., Mansooralikhan B. M., Srikumar B. N., Raju T. R., Kutty B. M., 2007. Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions. *Neuroscience.* 144, 412-423. <http://dx.doi.org/10.1016/j.neuroscience.2006.09.057>
- Birch, A. M., Kelly A. M., 2018. Lifelong environmental enrichment in the absence of exercise protects the brain from age-related cognitive decline. *Neuropharmacology.* <http://dx.doi.org/10.1016/j.neuropharm.2018.03.042>
- Birch, A. M., McGarry N. B., Kelly A. M., 2013. Short-term environmental enrichment, in the absence of exercise, improves memory, and increases NGF concentration, early neuronal survival, and synaptogenesis in the dentate gyrus in a time-dependent manner. *Hippocampus.* 23, 437-450. <http://dx.doi.org/10.1002/hipo.22103>
- Birnie, M., Morrison R., Camara R., Strauss K. I., 2013. Temporal changes of cytochrome P450 (Cyp) and eicosanoid-related gene expression in the rat brain after traumatic brain injury. *BMC Genomics.* 14, 303. <http://dx.doi.org/10.1186/1471-2164-14-303>
- Bishop, N. A., Lu T., Yankner B. A., 2010. Neural mechanisms of ageing and cognitive decline. *Nature.* 464, 529-535. <http://dx.doi.org/10.1038/nature08983>
- Brenes, J. C., Lackinger M., Hoglinger G. U., Schratz G., Schwarting R. K., Wöhr M., 2016. Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and

- ultrasonic communication in rats. *J Comp Neurol.* 524, 1586-1607.
<http://dx.doi.org/10.1002/cne.23842>
- Brinton, R. D., 2013. Neurosteroids as regenerative agents in the brain: therapeutic implications. *Nat Rev Endocrinol.* 9, 241-250. <http://dx.doi.org/10.1038/nrendo.2013.31>
- Cassilhas, R. C., Tufik S., de Mello M. T., 2016. Physical exercise, neuroplasticity, spatial learning and memory. *Cell Mol Life Sci.* 73, 975-983. <http://dx.doi.org/10.1007/s00018-015-2102-0>
- Charalampopoulos, I., Alexaki V. I., Tsatsanis C., Minas V., Dermitzaki E., Lasaridis I., et al., 2006. Neurosteroids as endogenous inhibitors of neuronal cell apoptosis in aging. *Ann N Y Acad Sci.* 1088, 139-152. <http://dx.doi.org/10.1196/annals.1366.003>
- Charalampopoulos, I., Remboutsika E., Margioris A. N., Gravanis A., 2008. Neurosteroids as modulators of neurogenesis and neuronal survival. *Trends Endocrinol Metab.* 19, 300-307. <http://dx.doi.org/10.1016/j.tem.2008.07.004>
- Compagnone, N. A., Mellon S. H., 2000. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol.* 21, 1-56.
<http://dx.doi.org/10.1006/frne.1999.0188>
- Cottrell, G. A., Nakajima S., 1977. Effect of corticosteroids in the hippocampus on passive avoidance behavior in the rat. *Pharmacol Biochem Behav.* 7, 277-280.
- Delchev, S., Georgieva K. (2018). *Cellular and Molecular Mechanisms of the Effects of Sex Hormones on the Nervous System.*
- Delgado-Morales, R., Agis-Balboa R. C., Esteller M., Berdasco M., 2017. Epigenetic mechanisms during ageing and neurogenesis as novel therapeutic avenues in human brain disorders. *Clin Epigenetics.* 9, 67. <http://dx.doi.org/10.1186/s13148-017-0365-z>
- Diederich, K., Bastl A., Wersching H., Teuber A., Strecker J. K., Schmidt A., et al., 2017. Effects of Different Exercise Strategies and Intensities on Memory Performance and Neurogenesis. *Front Behav Neurosci.* 11, 47. <http://dx.doi.org/10.3389/fnbeh.2017.00047>
- Dong, E., Matsumoto K., Uzunova V., Sugaya I., Takahata H., Nomura H., et al., 2001. Brain 5alpha-dihydroprogesterone and allopregnanolone synthesis in a mouse model of protracted social isolation. *Proc Natl Acad Sci U S A.* 98, 2849-2854.
<http://dx.doi.org/10.1073/pnas.051628598>
- Du, S. F., Yu Q., Chuan K., Ye C. L., He Z. J., Liu S. J., et al., 2017. In obese mice, exercise training increases 11beta-HSD1 expression, contributing to glucocorticoid activation and suppression of pulmonary inflammation. *J Appl Physiol* (1985). 123, 717-727.
<http://dx.doi.org/10.1152/japplphysiol.00652.2016>
- Faul, F., Erdfelder E., Lang A. G., Buchner A., 2007. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods.* 39, 175-191.
- Frick, K. M., Benoit J. D., 2010. Use it or lose it: environmental enrichment as a means to promote successful cognitive aging. *ScientificWorldJournal.* 10, 1129-1141.
<http://dx.doi.org/10.1100/tsw.2010.111>
- Frick, K. M., Fernandez S. M., 2003. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiol Aging.* 24, 615-626.
- Frick, K. M., Stearns N. A., Pan J. Y., Berger-Sweeney J., 2003. Effects of environmental enrichment on spatial memory and neurochemistry in middle-aged mice. *Learn Mem.* 10, 187-198.
<http://dx.doi.org/10.1101/lm.50703>
- Frye, C. A., 2009. Neurosteroids' effects and mechanisms for social, cognitive, emotional, and physical functions. *Psychoneuroendocrinology.* 34 Suppl 1, S143-161.
<http://dx.doi.org/10.1016/j.psyneuen.2009.07.005>

- Gasparoni, G., Bultmann S., Lutsik P., Kraus T. F. J., Sordon S., Vlcek J., et al., 2018. DNA methylation analysis on purified neurons and glia dissects age and Alzheimer's disease-specific changes in the human cortex. *Epigenetics Chromatin*. 11, 41. <http://dx.doi.org/10.1186/s13072-018-0211-3>
- Gresack, J. E., Kerr K. M., Frick K. M., 2007a. Life-long environmental enrichment differentially affects the mnemonic response to estrogen in young, middle-aged, and aged female mice. *Neurobiol Learn Mem*. 88, 393-408.
- Gresack, J. E., Kerr K. M., Frick K. M., 2007b. Short-term environmental enrichment decreases the mnemonic response to estrogen in young, but not aged, female mice. *Brain Res*. 1160, 91-101. <http://dx.doi.org/10.1016/j.brainres.2007.05.033>
- Griffin, E. W., Bechara R. G., Birch A. M., Kelly A. M., 2009. Exercise enhances hippocampal-dependent learning in the rat: evidence for a BDNF-related mechanism. *Hippocampus*. 19, 973-980. <http://dx.doi.org/10.1002/hipo.20631>
- Harburger, L. L., Lambert T. J., Frick K. M., 2007. Age-dependent effects of environmental enrichment on spatial reference memory in male mice. *Behav Brain Res*. 185, 43-48. <http://dx.doi.org/10.1016/j.bbr.2007.07.009>
- Higo, S., Hojo Y., Ishii H., Kominami T., Nakajima K., Poirier D., et al., 2009. Comparison of sex-steroid synthesis between neonatal and adult rat hippocampus. *Biochem Biophys Res Commun*. 385, 62-66. <http://dx.doi.org/10.1016/j.bbrc.2009.05.005>
- Higuchi, R., Fockler C., Dollinger G., Watson R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)*. 11, 1026-1030.
- Hojo, Y., Higo S., Kawato S., Hatanaka Y., Ooishi Y., Murakami G., et al., 2011. Hippocampal synthesis of sex steroids and corticosteroids: essential for modulation of synaptic plasticity. *Front Endocrinol (Lausanne)*. 2, 43. <http://dx.doi.org/10.3389/fendo.2011.00043>
- Hojo, Y., Kawato S., 2018. Neurosteroids in Adult Hippocampus of Male and Female Rodents: Biosynthesis and Actions of Sex Steroids. *Front Endocrinol (Lausanne)*. 9, 183. <http://dx.doi.org/10.3389/fendo.2018.00183>
- Howard, B., Wang Y., Xekouki P., Faucz F. R., Jain M., Zhang L., et al., 2014. Integrated analysis of genome-wide methylation and gene expression shows epigenetic regulation of CYP11B2 in aldosteronomas. *J Clin Endocrinol Metab*. 99, E536-543. <http://dx.doi.org/10.1210/jc.2013-3495>
- Ibanez, C., Guennoun R., Liere P., Eychenne B., Pianos A., El-Etr M., et al., 2003. Developmental expression of genes involved in neurosteroidogenesis: 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase in the rat brain. *Endocrinology*. 144, 2902-2911. <http://dx.doi.org/10.1210/en.2002-0073>
- Kass, L., Altamirano G. A., Bosquiaz V. L., Luque E. H., Munoz-de-Toro M., 2012. Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in Wistar rats. *Reprod Toxicol*. 33, 390-400. <http://dx.doi.org/10.1016/j.reprotox.2012.02.002>
- Kelemen, E., Bahrendt M., Born J., Inostroza M., 2014. Hippocampal corticosterone impairs memory consolidation during sleep but improves consolidation in the wake state. *Hippocampus*. 24, 510-515. <http://dx.doi.org/10.1002/hipo.22266>
- Kim, H. J., Park C. H., Roh G. S., Kang S. S., Cho G. J., Choi W. S., 2002. Changes of steroidogenic acute regulatory protein mRNA expression in postnatal rat development. *Brain Res Dev Brain Res*. 139, 247-254.
- Kimoto, T., Ishii H., Higo S., Hojo Y., Kawato S., 2010. Semicomprehensive analysis of the postnatal age-related changes in the mRNA expression of sex steroidogenic enzymes and sex steroid

- receptors in the male rat hippocampus. *Endocrinology*. 151, 5795-5806.
<http://dx.doi.org/10.1210/en.2010-0581>
- Lambert, T. J., Fernandez S. M., Frick K. M., 2005. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. *Neurobiol Learn Mem.* 83, 206-216. <http://dx.doi.org/10.1016/j.nlm.2004.12.001>
- Lardenoije, R., Iatrou A., Kenis G., Kompotis K., Steinbusch H. W., Mastroeni D., et al., 2015. The epigenetics of aging and neurodegeneration. *Prog Neurobiol.* 131, 21-64.
<http://dx.doi.org/10.1016/j.pneurobio.2015.05.002>
- Lavaque, E., Mayen A., Azcoitia I., Tena-Sempere M., Garcia-Segura L. M., 2006. Sex differences, developmental changes, response to injury and cAMP regulation of the mRNA levels of steroidogenic acute regulatory protein, cytochrome p450scc, and aromatase in the olivocerebellar system. *J Neurobiol.* 66, 308-318. <http://dx.doi.org/10.1002/neu.20221>
- Laviola, G., Hannan A. J., Macri S., Solinas M., Jaber M., 2008. Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiol Dis.* 31, 159-168. <http://dx.doi.org/10.1016/j.nbd.2008.05.001>
- Lazzarino, G. P., Andreoli M. F., Rossetti M. F., Stoker C., Tschopp M. V., Luque E. H., et al., 2017. Cafeteria diet differentially alters the expression of feeding-related genes through DNA methylation mechanisms in individual hypothalamic nuclei. *Mol Cell Endocrinol.* 450, 113-125. <http://dx.doi.org/10.1016/j.mce.2017.05.005>
- Leal-Galicia, P., Castaneda-Bueno M., Quiroz-Baez R., Arias C., 2008. Long-term exposure to environmental enrichment since youth prevents recognition memory decline and increases synaptic plasticity markers in aging. *Neurobiol Learn Mem.* 90, 511-518.
<http://dx.doi.org/10.1016/j.nlm.2008.07.005>
- Luchetti, S., Bossers K., Van de Bilt S., Agrapart V., Morales R. R., Frajese G. V., et al., 2011a. Neurosteroid biosynthetic pathways changes in prefrontal cortex in Alzheimer's disease. *Neurobiol Aging.* 32, 1964-1976. <http://dx.doi.org/10.1016/j.neurobiolaging.2009.12.014>
- Luchetti, S., Huitinga I., Swaab D. F., 2011b. Neurosteroid and GABA-A receptor alterations in Alzheimer's disease, Parkinson's disease and multiple sclerosis. *Neuroscience.* 191, 6-21.
<http://dx.doi.org/10.1016/j.neuroscience.2011.04.010>
- Luine, V. N., 2014. Estradiol and cognitive function: past, present and future. *Horm Behav.* 66, 602-618. <http://dx.doi.org/10.1016/j.yhbeh.2014.08.011>
- Lupien, S. J., Lepage M., 2001. Stress, memory, and the hippocampus: can't live with it, can't live without it. *Behav Brain Res.* 127, 137-158.
- Martinez-Arguelles, D. B., Papadopoulos V., 2010. Epigenetic regulation of the expression of genes involved in steroid hormone biosynthesis and action. *Steroids.* 75, 467-476.
<http://dx.doi.org/10.1016/j.steroids.2010.02.004>
- Mellon, S. H., 2007. Neurosteroid regulation of central nervous system development. *Pharmacol Ther.* 116, 107-124. <http://dx.doi.org/10.1016/j.pharmthera.2007.04.011>
- Mellon, S. H., Gong W., Schonemann M. D., 2008. Endogenous and synthetic neurosteroids in treatment of Niemann-Pick Type C disease. *Brain Res Rev.* 57, 410-420.
<http://dx.doi.org/10.1016/j.brainresrev.2007.05.012>
- Micheau, J., Destrade C., Soumireu-Mourat B., 1984. Time-dependent effects of posttraining intrahippocampal injections of corticosterone on retention of appetitive learning tasks in mice. *Eur J Pharmacol.* 106, 39-46.
- Missaghian, E., Kempna P., Dick B., Hirsch A., Alikhani-Koupaei R., Jegou B., et al., 2009. Role of DNA methylation in the tissue-specific expression of the CYP17A1 gene for steroidogenesis in rodents. *J Endocrinol.* 202, 99-109. <http://dx.doi.org/10.1677/JOE-08-0353>

- Moore, L. D., Le T., Fan G., 2013. DNA methylation and its basic function. *Neuropsychopharmacology*. 38, 23-38. <http://dx.doi.org/10.1038/npp.2012.112>
- Mora, F., Segovia G., del Arco A., 2007. Aging, plasticity and environmental enrichment: structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Res Rev*. 55, 78-88. <http://dx.doi.org/10.1016/j.brainresrev.2007.03.011>
- Moreno-Piovan, G. S., Varayoud J., Luque E. H., Ramos J. G., 2014. Long-term ovariectomy increases BDNF gene methylation status in mouse hippocampus. *J Steroid Biochem Mol Biol*. 144 Pt B, 243-252. <http://dx.doi.org/10.1016/j.jsbmb.2014.08.001>
- Munetsuna, E., Hattori M., Komatsu S., Sakimoto Y., Ishida A., Sakata S., et al., 2009. Social isolation stimulates hippocampal estradiol synthesis. *Biochem Biophys Res Commun*. 379, 480-484. <http://dx.doi.org/10.1016/j.bbrc.2008.12.076>
- Munetsuna, E., Hattori M., Sakimoto Y., Ishida A., Sakata S., Hojo Y., et al., 2011. Environmental enrichment alters gene expression of steroidogenic enzymes in the rat hippocampus. *Gen Comp Endocrinol*. 171, 28-32. <http://dx.doi.org/10.1016/j.ygcen.2010.12.007>
- Nativio, R., Donahue G., Berson A., Lan Y., Amlie-Wolf A., Tuzer F., et al., 2018. Dysregulation of the epigenetic landscape of normal aging in Alzheimer's disease. *Nat Neurosci*. 21, 497-505. <http://dx.doi.org/10.1038/s41593-018-0101-9>
- Nithianantharajah, J., Hannan A. J., 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat Rev Neurosci*. 7, 697-709. <http://dx.doi.org/10.1038/nrn1970>
- Nomura, M., Morohashi K., Kirita S., Nonaka Y., Okamoto M., Nawata H., et al., 1993. Three forms of rat CYP11B genes: 11 beta-hydroxylase gene, aldosterone synthase gene, and a novel gene. *J Biochem*. 113, 144-152.
- Rapley, S. A., Prickett T. C. R., Dalrymple-Alford J. C., Espiner E. A., 2018. Environmental Enrichment Elicits a Transient Rise of Bioactive C-Type Natriuretic Peptide in Young but Not Aged Rats. *Front Behav Neurosci*. 12, 142. <http://dx.doi.org/10.3389/fnbeh.2018.00142>
- Reddy, D. S., 2010. Neurosteroids: endogenous role in the human brain and therapeutic potentials. *Prog Brain Res*. 186, 113-137. <http://dx.doi.org/10.1016/B978-0-444-53630-3.00008-7>
- Roselli, C. F., 2007. Brain aromatase: roles in reproduction and neuroprotection. *J Steroid Biochem Mol Biol*. 106, 143-150. <http://dx.doi.org/10.1016/j.jsbmb.2007.05.014>
- Rosenzweig, M. R., Bennett E. L., Hebert M., Morimoto H., 1978. Social grouping cannot account for cerebral effects of enriched environments. *Brain Res*. 153, 563-576.
- Rossetti, M. F., Cambiasso M. J., Holschbach M. A., Cabrera R., 2016a. Oestrogens and Progestagens: Synthesis and Action in the Brain. *J Neuroendocrinol*. 28. <http://dx.doi.org/10.1111/jne.12402>
- Rossetti, M. F., Varayoud J., Andreoli M. F., Stoker C., Luque E. H., Ramos J. G., 2018. Sex- and age-associated differences in episodic-like memory and transcriptional regulation of hippocampal steroidogenic enzymes in rats. *Mol Cell Endocrinol*. 470, 208-218. <http://dx.doi.org/10.1016/j.mce.2017.11.001>
- Rossetti, M. F., Varayoud J., Lazzarino G. P., Luque E. H., Ramos J. G., 2016b. Pregnancy and lactation differentially modify the transcriptional regulation of steroidogenic enzymes through DNA methylation mechanisms in the hippocampus of aged rats. *Mol Cell Endocrinol*. 429, 73-83. <http://dx.doi.org/10.1016/j.mce.2016.03.037>
- Rossetti, M. F., Varayoud J., Moreno-Piovan G. S., Luque E. H., Ramos J. G., 2015. Environmental enrichment attenuates the age-related decline in the mRNA expression of steroidogenic enzymes and reduces the methylation state of the steroid 5alpha-reductase type 1 gene in the rat hippocampus. *Mol Cell Endocrinol*. <http://dx.doi.org/10.1016/j.mce.2015.05.024>

- Rune, G. M., Frotscher M., 2005. Neurosteroid synthesis in the hippocampus: role in synaptic plasticity. *Neuroscience*. 136, 833-842.
<http://dx.doi.org/10.1016/j.neuroscience.2005.03.056>
- Ryan, S. M., Kelly A. M., 2016. Exercise as a pro-cognitive, pro-neurogenic and anti-inflammatory intervention in transgenic mouse models of Alzheimer's disease. *Ageing Res Rev.* 27, 77-92. <http://dx.doi.org/10.1016/j.arr.2016.03.007>
- Sager, T., Kashon M. L., Krajnak K., 2018. Estrogen and Environmental Enrichment Differentially Affect Neurogenesis, Dendritic Spine Immunolabeling and Synaptogenesis in the Hippocampus of Young and Reproductively Senescent Female Rats. *Neuroendocrinology*. 106, 252-263. <http://dx.doi.org/10.1159/000479699>
- Sale, A., Berardi N., Maffei L., 2009. Enrich the environment to empower the brain. *Trends Neurosci.* 32, 233-239. <http://dx.doi.org/10.1016/j.tins.2008.12.004>
- Schumacher, M., Weill-Engerer S., Liere P., Robert F., Franklin R. J., Garcia-Segura L. M., et al., 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog Neurobiol.* 71, 3-29.
- Singh, C., Liu L., Wang J. M., Irwin R. W., Yao J., Chen S., et al., 2012. Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice. *Neurobiol Aging*. 33, 1493-1506.
<http://dx.doi.org/10.1016/j.neurobiolaging.2011.06.008>
- Sleiman, S. F., Henry J., Al-Haddad R., El Hayek L., Abou Haidar E., Stringer T., et al., 2016. Exercise promotes the expression of brain derived neurotrophic factor (BDNF) through the action of the ketone body beta-hydroxybutyrate. *Elife*. 5. <http://dx.doi.org/10.7554/eLife.15092>
- Stein, L. R., O'Dell K. A., Funatsu M., Zorumski C. F., Izumi Y., 2016. Short-term environmental enrichment enhances synaptic plasticity in hippocampal slices from aged rats. *Neuroscience*. 329, 294-305. <http://dx.doi.org/10.1016/j.neuroscience.2016.05.020>
- Valero, J., Espana J., Parra-Damas A., Martin E., Rodriguez-Alvarez J., Saura C. A., 2011. Short-term environmental enrichment rescues adult neurogenesis and memory deficits in APP(Sw,Ind) transgenic mice. *PLoS One*. 6, e16832. <http://dx.doi.org/10.1371/journal.pone.0016832>
- van Praag, H., Kempermann G., Gage F. H., 2000. Neural consequences of environmental enrichment. *Nat Rev Neurosci.* 1, 191-198. <http://dx.doi.org/10.1038/35044558>
- Vanselow, J., Pohland R., Furbass R., 2005. Promoter-2-derived Cyp19 expression in bovine granulosa cells coincides with gene-specific DNA hypo-methylation. *Mol Cell Endocrinol.* 233, 57-64. <http://dx.doi.org/10.1016/j.mce.2005.01.007>
- Vanselow, J., Spitschak M., Nimz M., Furbass R., 2010. DNA methylation is not involved in preovulatory down-regulation of CYP11A1, HSD3B1, and CYP19A1 in bovine follicles but may have a role in permanent silencing of CYP19A1 in large granulosa lutein cells. *Biol Reprod.* 82, 289-298. <http://dx.doi.org/10.1095/biolreprod.109.079251>
- Varayoud, J., Ramos J. G., Bosquiaz V. L., Munoz-de-Toro M., Luque E. H., 2008. Developmental exposure to Bisphenol a impairs the uterine response to ovarian steroids in the adult. *Endocrinology*. 149, 5848-5860. <http://dx.doi.org/10.1210/en.2008-0651>
- Yang, S., Fang Z., Suzuki T., Sasano H., Zhou J., Gurates B., et al., 2002. Regulation of aromatase P450 expression in endometriotic and endometrial stromal cells by CCAAT/enhancer binding proteins (C/EBPs): decreased C/EBPbeta in endometriosis is associated with overexpression of aromatase. *J Clin Endocrinol Metab.* 87, 2336-2345.
<http://dx.doi.org/10.1210/jcem.87.5.8486>
- Yoshii, Y., Oki K., Gomez-Sanchez C. E., Ohno H., Itcho K., Kobuke K., et al., 2016. Hypomethylation of CYP11B2 in Aldosterone-Producing Adenoma. *Hypertension*. 68, 1432-1437.
<http://dx.doi.org/10.1161/HYPERTENSIONAHA.116.08313>

Zhang, X., Ho S. M., 2011. Epigenetics meets endocrinology. *J Mol Endocrinol.* 46, R11-32.

Legends

Table 1. The sequences of primer oligonucleotides for PCR amplification.

Table 2. Antibodies used for immunohistochemistry.

Figure 1. Pathway of neurosteroid synthesis in the rat hippocampus. Steroidogenic acute regulatory protein (StAR); cytochrome P450 side chain cleavage (P450scc); 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD); cytochrome P450 17 α -hydroxylase/c17,20-lyase (P450(17 α)); steroid 5 α -reductase (5 α R); 3 α -hydroxysteroid dehydrogenase (3 α -HSD); cytochrome. P4502d4 (P450(2d4)); 11 β -hydroxylase (P450(11 β)-1); aldosterone synthase (P450(11 β)-2); 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and cytochrome P450 aromatase (P450arom).

Figure 2. Experimental protocol and caging conditions. Rats were maintained under standard laboratory conditions (SC) from postnatal day (PND) 0 to PND 80 (Y-SC) or to PND 350 (A-SC). At PND 80 and 350, animals (n=16/group) were left in standard conditions (SC; n=8/cage) or differentially housed in a sensory enriched environment (SE; n=8/cage) or a motor enriched environment (ME; n=8/cage) up to PND 90 (Y) and 360 (A).

Figure 3. Characterization of P450arom and 5 α R-1 antisera using rat ovary. A) In Western blot assays, native proteins (58 kDa and 29 kDa, respectively) were detected in the ovary. Specific protein detection was negative when primary antibodies were absent (NC). B)

Immunoreactivity of the antibodies was evaluated by immunohistochemistry in tissue sections of ovary. (a) Specific P450arom immunoreactivity was observed as a speckled cytoplasmic pattern compatible with a mitochondrial located protein in the cytoplasm of oocytes. (b) Positive staining for 5 α R-1 was found in the cytoplasm of the ovarian granulosa and theca cells. (c, d) Specific staining was absent when the primary antibodies were preabsorbed with the peptide used as immunogen. The reactions were developed using diaminobenzidine and counterstained with Mayers's hematoxylin. Scale bars: 50 μ m. Steroid 5 α -reductase type 1 (5 α R-1); cytochrome P450 aromatase (P450arom).

Figure 4. Real-time PCR analysis of the mRNA levels of steroidogenic enzymes and StAR in young adult (Y) and middle-aged (A) female rat hippocampus under sensory and motor enrichment (SE and ME) versus standard conditions (SC). The amounts of mRNA in Y-SC, Y-SE, Y-ME, A-SE and A-ME rats are indicated as relative values to A-SC. The columns and error bars represent the means \pm SEM (n=8/group). Different letters indicate a significant difference at $p < 0.05$ by Bonferroni's test after two-way ANOVA.

Figure 5. Immunohistochemistry analysis of P450arom and 5 α R-1 antisera in rat hippocampus. Young adult (Y) and middle-aged (A) female animals housed under standard laboratory conditions (SC) and exposed to sensory and motor enrichment for 10 days (SE and ME, respectively) were analyzed. In CA1-CA3 and dentate gyrus (DG) areas, specific immunoreaction was observed in the cytoplasm of neurons as speckled-labeling pattern (black arrows). Scale bars: 300 μ m; the inset shows higher magnification of the clusters in DG, although the pattern was similar in all studied areas. Steroid 5 α -reductase type 1 (5 α R-1); cytochrome P450 aromatase (P450arom).

871

872 **Figure 6.** Map of the P450(17 α) promoter. The positions of the TATA box are indicated.
873 CpG islands and CG target sites for digestion by the methylation-sensitive restriction
874 enzyme *Mae II* (ACGT) are indicated.

875

876 **Figure 7.** Methylation analysis using methylation-sensitive restriction enzymes followed
877 by real-time PCR in the female hippocampus of young adult (Y) and middle-aged (A) rats
878 housed under standard laboratory conditions (SC) and exposed to sensory and motor
879 enrichment for 10 days (SE and ME, respectively). Methylation-sensitive restriction sites of
880 the 3 α -HSD (A), 5 α R-1(B), P450(17 α) (C), P450(11 β)-2 (D) and P450arom (E) gene
881 promoters were studied. The relative methylation state in Y-SC, Y-SE, Y-ME, A-SE and
882 A-ME rats are indicated as relative values to A-SC. The columns and error bars represent
883 the means \pm SEM (n=8/group). Different letters indicate a significant difference at $p < 0.05$
884 by Bonferroni's test after two-way ANOVA.

885

Table 1. The sequences of primer oligonucleotides for PCR amplification.

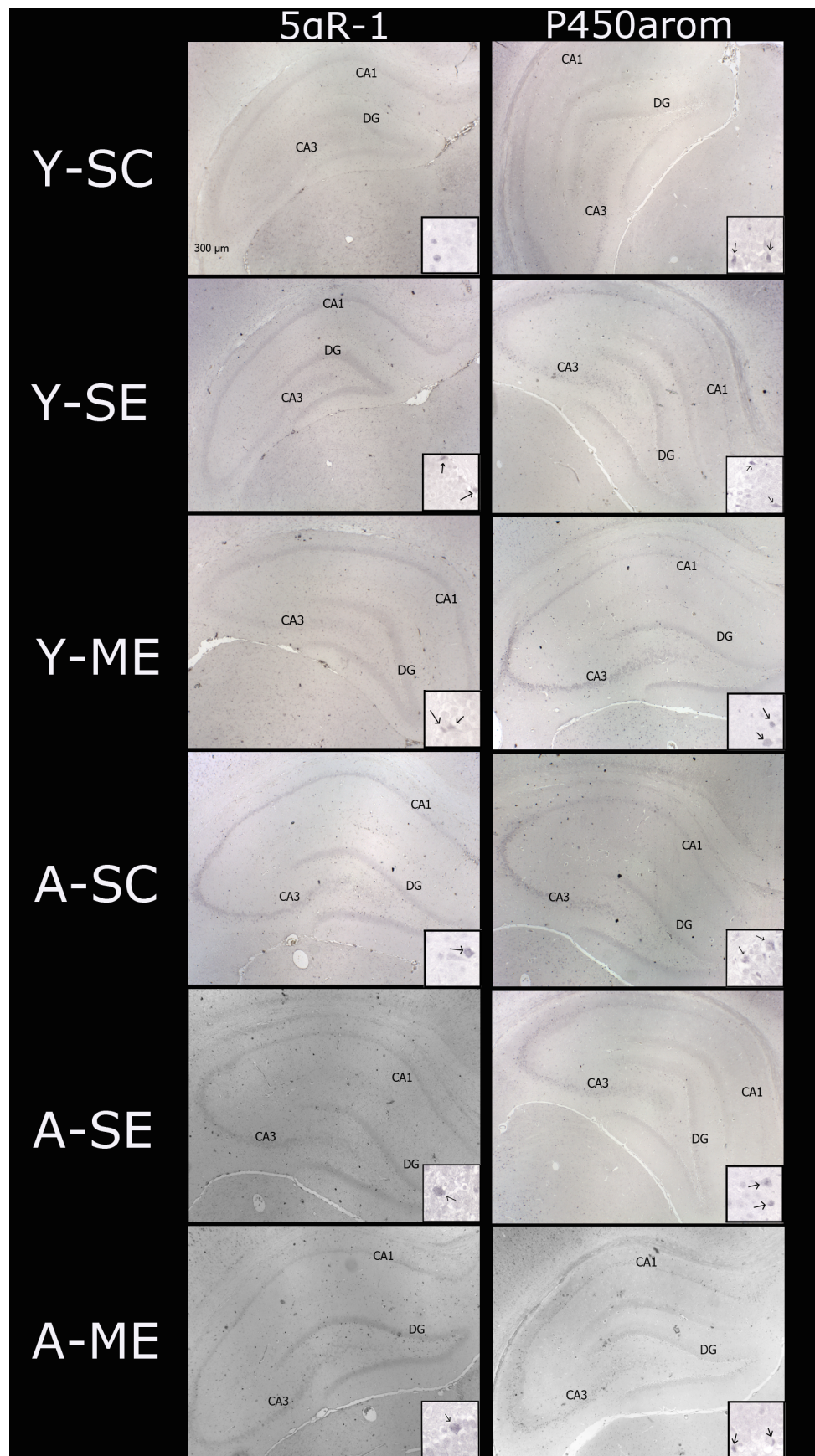
Targets	Primer sense	Primer antisense
IC P450(17 α)	5'- GCAACTGAACTAAAACAAGG - 3'	5'- TAAGCAACAACCTCTCCAATC - 3'
<i>Mae II</i> (a) P450(17 α)	5'- AGGAGTATTCATAGGCAGAA - 3'	5'- GTATAAATCTTGTGGGCAAC - 3'
<i>Mae II</i> (b) P450(17 α)	5'- CAGAGAGATGGCACAAATGT - 3'	5'- TACATGCAGGTAAAAGGCTC - 3'

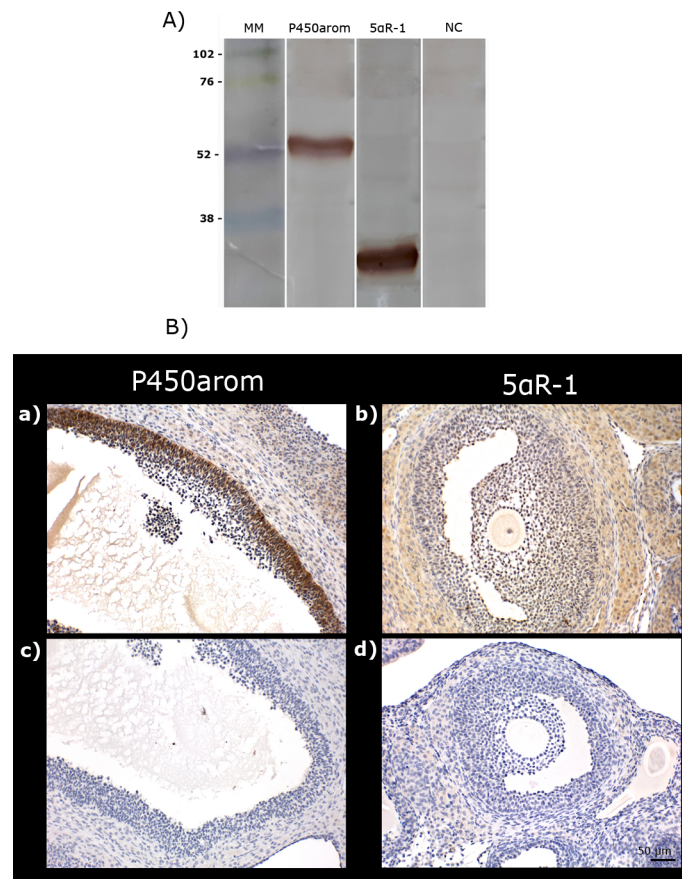
IC: internal control; P450(17 α): cytochrome P450 17 α -hydroxylase/c17,20-lyase.

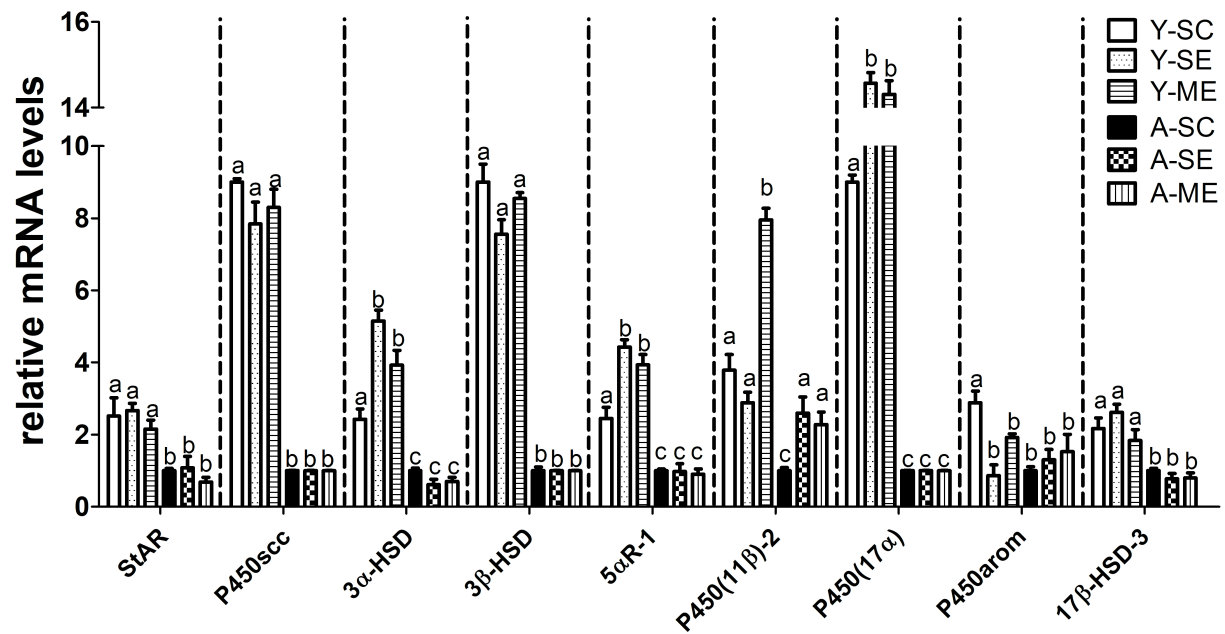
Table 2. Antibodies used for immunohistochemistry.

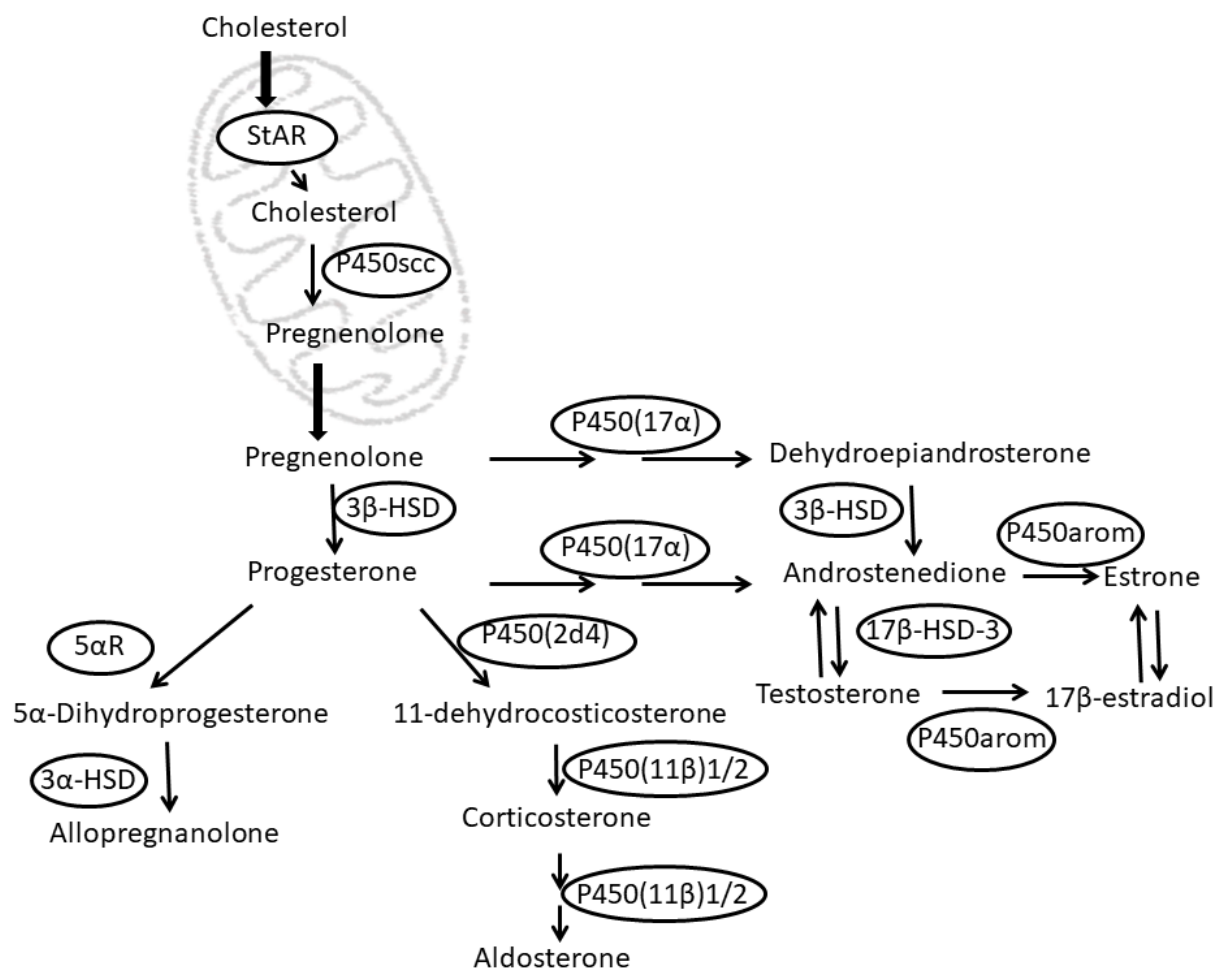
Antibody	Dilution	Supplier
Primary		
Anti- 5 α R-1	1/500	Instituto de Salud y Ambiente del Litoral (Santa Fe, Argentina)*
Anti-P450arom	1/500	Instituto de Salud y Ambiente del Litoral (Santa Fe, Argentina)*
Secondary		
Anti-rabbit	1/200	Sigma (St. Louis, MO)

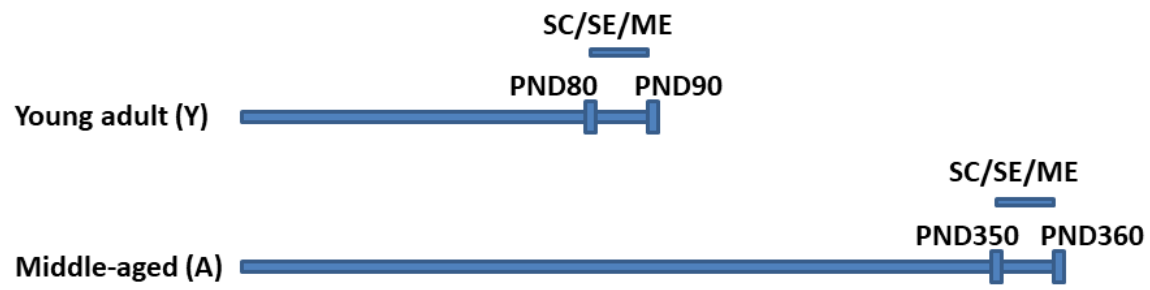
* Anti-5 α R-1 and Anti-P450arom were generated and tested in our laboratory as was described by Varayoud et al. (2008). 5 α R-1: steroid 5 α Reductase type 1; P450arom: cytochrome P450 aromatase.











STANDARD CONDITION (SC)



SENSORY ENRICHMENT (SE)



MOTOR ENRICHMENT (ME)

