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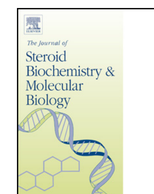

# Long-term ovariectomy increases *BDNF* gene methylation status in mouse hippocampus

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ABSTRACT

Estradiol (E) has been suggested to have a neuroprotective effect in young animals but has neutral or harmful effects when it is administered to aged animals. In the present study, we determined whether the post-ovariectomy (post-OVX) timeframe elapsed before the initiation of chronic E treatment is critical for the estrogenic induction of neurotrophins (brain-derived neurotrophic factor, BDNF, and synaptophysin, SYN) in the rodent hippocampus. Adult mice were OVX and, a short period (short-term E (STE) animals) or a long period (long-term E (LTE) animals) after the OVX, were daily treated with E. Control animals were treated with sesame oil (short-term control (STC) and long-term control (LTC) animals). Protein expression was determined using an immunohistochemical approach. Transcriptional activity in the hippocampus of individual BDNF promoters was assessed by real-time quantitative RT-PCR, and the methylation levels of regulatory regions were analyzed by methylation-specific PCR and combined bisulfite restriction analysis. STE animals showed increased BDNF and SYN protein expression and a higher activity of BDNF II, IV, and V promoters. In contrast, LTE animals did not show E induction of neurotrophins. In these animals, the methylation levels of regulatory sequences of the BDNF were higher than in the STE animals in a CpG island of promoter V and in the CRE regulatory site located in promoter IV. With this experiment, we determined that a prolonged period of hypoestrogenicity disrupts the E-induction of neurotrophins, and we postulated that DNA methylation is one of the epigenetic mechanisms that could explain the E-insensitivity of the BDNF after a long period post-OVX.

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## 1. Introduction

Estrogens influence the developing nervous system and stimulate the normal functioning of neurons in adulthood. Diminished estrogen plasma levels due to surgical or natural menopause have been associated with cognitive decline [1], while estrogen replacement decreases the risk for Alzheimer's disease (AD) [2] and stroke [3]. The 'critical period' hypothesis has been proposed in an attempt to clarify the discrepant findings on estrogen and cognitive functioning in women. According to this theory, estrogen therapy effectively decreases the cognitive decline associated with normal aging if the treatment is initiated at the time of menopause or very early in the postmenopausal period and is continued for several years thereafter. Furthermore, estrogen therapy has no effect, or might even cause harm, when treatment is initiated decades after the menopausal transition. Indeed, evidence from basic neuroscience and animal behavior studies supports the idea that estradiol has a neuroprotective effect in young animals but has neutral or harmful effects when administered to aged animals [4].

The sites of action of estrogen on cognitive performance have not been fully established, but one probable site is the hippocampus, a steroid-responsive brain region [5,6]. The influence of 17β-estradiol (E) on cognition and mood could occur via the regulation of neurotrophic factors, such as brain-derived

*Abbreviations:* BDNF, brain-derived neurotrophic factor; COBRA, combined bisulfite restriction analysis; DG, dentate gyrus; E, 17β-estradiol; HRT, hormone replacement therapy; IOD, integrated optical density; QMSP, real-time quantitative methylation-specific PCR; PZ, polymorphic zone; SYN, synaptophysin; QPCR, real-time quantitative PCR; WHIMS, Women's Health Initiative Memory Study.

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neurotrophic factor (BDNF), which affects neuronal survival and synaptic plasticity [7,8]. BDNF has been implicated in learning and memory [9,10], Alzheimer's disease [11], and psychosis [12]. Another way in which estrogen may improve mnemonic function is by enhancing synaptic plasticity in the hippocampus. For example, increases in synaptophysin (SYN), a presynaptic protein located in the membranes of neurotransmitter-containing vesicles [13] have been noted following estrogen treatment. Furthermore, estrogen increases levels of SYN in aged female mice, a finding which has been associated with enhanced hippocampal-dependent learning [14].

Several studies have examined the effect of E on BDNF expression in the hippocampal formation of female rodents [15]. On the morning of proestrus, BDNF protein was elevated relative to ovariectomized rats, and it was elevated in the mossy fibers in particular, suggesting that estradiol elevated the level of BDNF in the same areas where it is normally expressed, rather than ectopic locations. Interestingly, BDNF levels were also elevated in the mossy fibers on the morning of estrus and finally decreasing on the morning of metestrus [16]. These results were replicated using ovariectomized rats that were treated with estradiol to simulate proestrous morning [17].

Long-term (25 weeks) E replacement in aged ovariectomized (OVX) rats restored *BDNF* mRNA levels in some hippocampal subfields [18], however, other studies of short-term E treatment have reported decreased BDNF protein [19], no change [15], or increased *BDNF* mRNA [20]. These apparently discordant results could be explained by differences in the experimental designs utilized in each work. Differences in the post-ovariectomy (post-OVX) timeframe elapsed before E replacement initiation have been recognized as a source of variability in E-induced BDNF experiments [21]. Additionally, some doses and regimens of estrogen may simply fail to influence BDNF [21]. Berchtold et al. [22] demonstrated that physical activity increased *BDNF* mRNA in the hippocampus after short-term (3 weeks) estrogen deprivation. However, long-term estrogen deprivation blunted the exercise effect, as in 7-week OVX rats, exercise alone no longer affected either mRNA or protein levels of BDNF in the hippocampus. This loss of brain responsiveness to many neurotrophic stimuli in long-lasting OVX animals or in women during advanced menopause has been reported by many authors [23,24], but the molecular events associated with this phenomenon have not been elucidated. In summary these studies suggest that estradiol can induce *BDNF* gene expression in the hippocampal formation under physiological and non-physiological conditions, i.e., after OVX.

The mechanisms underlying E-dependent regulation of hippocampal BDNF expression are unknown, due in part to the complicated structure of the *BDNF*. The gene contains at least five short 5′-non-coding exons in mice, each of which can be alternatively spliced to a common coding exon VI to form several mRNA transcripts, all coding for an identical BDNF protein [25]. An estrogen response element (ERE)-like sequence has been described in the *BDNF*, but little is known about its functional significance under in vivo conditions [26]. Each non-coding exon of the *BDNF* gene is preceded by a specific promoter region that regulates transcription in a tissue-specific fashion. This transcription control is driven through the regulatory action of multiple *cis*-acting motifs that contain CpG-rich sequences. It has been demonstrated that increased synthesis of BDNF in neurons after depolarization correlates with a decrease in CpG methylation within regulatory regions of the *BDNF* [27,28].

In the present work, we investigated whether the post-OVX timeframe elapsed before hormone replacement therapy (HRT) initiation is critical for the E-mediated induction of neurotrophic proteins (BDNF and SYN) and estrogen receptor alpha (ERalpha) expression. In addition, to better understand the molecular mechanisms of E effects, we particularly studied both the relative abundance of total *BDNF* mRNA, including the transcripts with alternative 5′ UTR exons, and the methylation status of the *BDNF*.

## 2. Materials and methods

### 2.1. Animals

All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina) and were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences. Seventy-two female Balb/c mice from our in-house colony (Department of Physiology, University of Litoral) were selected. At the beginning of the experiment, the animals were 10 weeks of age and weighed 15–20 g. Mice were housed in pairs, under controlled conditions, with a 12/12 h light/dark cycle (lights on 0700 h) and food (Cooperación, Santa Fe, Argentina) and water ad libitum. Temperature and humidity were maintained between $22 \pm 2\,^{\circ}$C and 50–55%, respectively, during all experiments.

### 2.2. Experimental paradigm

The experimental design is schematized in Fig. 1. Seventy-two mice were randomly assigned to four different groups (18 animals/group): long-term OVX controls (LTC), long-term OVX treated with estradiol (LTE), short-term OVX controls (STC), and short-term OVX treated with estradiol (STE). In the first two groups (LTC and LTE), OVX was performed at 3 months of age, and the hormonal treatment began when animals were 10 months of age. The hormonal replacement consisted of a daily s.c. injection of E (0.033 mg E/kg of body weight in STE and LTE groups) or vehicle (50 μl/animal of sesame oil in STC and LTC groups) for 2 weeks (chronic estrogen treatment). To determine the E serum levels, we obtained blood samples 4 h after vehicle (STC and LTC animals) or E injection (STE and LTE animals).

In the STC and STE groups, animals were OVX at 10 months of age and treated with estradiol (STE) or vehicle (STC) 2 weeks after the OVX for 15 days, with the same regime described above. This experimental approach not intended to be an experimental model of menopause but allowed us to evaluate the effects of two very different timeframes between ovarian function depletion (OVX) and estrogen treatment initiation (15 days in the STE animals vs. 7.5 months in the LTE group), on hippocampal BDNF expression. Moreover, all of the experimental animals received the estrogen treatment and were sacrificed at exactly the same age (10.5 and 11 months for the HRT and sacrifice, respectively), eliminating confounding effects observed in previous reports [29].

Twenty-four hours after the last injection, animals were sacrificed by decapitation. For immunohistochemical procedures, brains from 6 animals of each experimental group were fixed by immersion in 4% paraformaldehyde in PBS for 48 h at 4 °C. The fixed tissue was dehydrated in an ascending ethanol series, cleared in xylene and embedded in paraffin. For molecular biology techniques, a second set of brains ($n = 12$ per treatment group) was quickly removed, and the whole hippocampus (including the dentate gyrus, DG) was microdissected. Tissue blocks were flash-frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until DNA or RNA extraction.

### 2.3. Hormone assays

Blood serum samples were obtained for hormone assays. Serum levels of E were determined by RIA after ethyl ether and hexane

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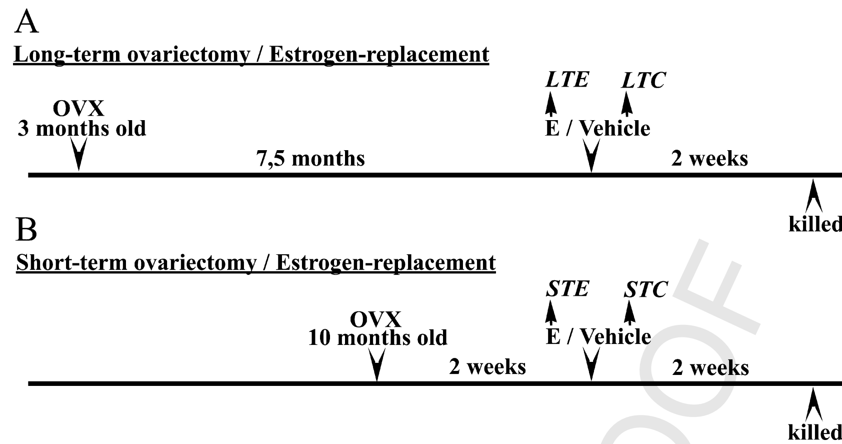


**Fig. 1.** Schematic representation of experimental protocol. OVX, ovariectomy; E, 17β-estradiol. LTC and LTE, long-term OVX controls and long-term OVX treated with estradiol animals, respectively. STC and STE, short-term OVX controls and treated with estradiol animals, respectively.

(Merck, Buenos Aires, Argentina) extraction, respectively [30]. The antibodies were provided by G.D. Niswender, and labeled hormones were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Assay sensitivity was 1.6 pg/ml and intra- and interassay coefficients of variation were 3.6 and 11%.

### *2.4. RNA extraction, reverse transcription, and real-time quantitative PCR analysis (QPCR)*

An optimized RT-PCR protocol was employed to analyze the relative expression levels of each of five transcripts of the *BDNF* containing different 5′-non-coding exons (exons I–V). Another set of primers complementary to the common exon VI of all BDNF transcripts was designed to measure levels of total *BDNF* mRNA (Table 1). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was assessed by $A_{260}$, and RNA was stored at −80 °C until needed. Equal quantities (4 μg) of total RNA were reverse-transcribed into cDNA with MMLV reverse transcriptase (300 U; Promega, Madison, WI, USA) using 200 pmol of random primers (Promega). Twenty units of ribonuclease inhibitor (RNAout) (Invitrogen) and 100 nmol of a deoxy-NTP mixture were added to each reaction tube in a final volume of 30 μl of 1X MMLV-RT buffer. Reverse transcription was performed at 42 °C for 90 min. Reactions were terminated by heating at 97 °C for 5 min and cooling on ice, followed by dilution of the reverse-transcribed cDNA with RNase-free water to a final volume of 60 μl. Samples were analyzed in triplicate, and a sample without reverse transcriptase was included to detect contamination by genomic DNA. cDNA levels were detected using QPCR with the DNA Engine Opticon System (Bio-Rad Laboratories Inc., Waltham, MA, USA) and SYBR Green I dye (Cambrex Corp., East Rutherford, NJ, USA). For cDNA amplification, 5 μl of cDNA was combined with a mixture containing 2.5 U *Taq*-DNA polymerase (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), and 10 pmol of each primer (Invitrogen) in a final volume of 25 μl of 1X SYBR Green I PCR *Taq* buffer. After initial denaturation at 97 °C for 1 min, the reaction mixture was subjected to successive cycles of denaturation at 97 °C for 15 s, annealing at 55 °C (for all BDNF transcript variants) or 57 °C (for 18S rRNA) 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 lacking a DNA template were included in all of the assays, yielding no consistent amplification. All PCR products were cloned using a TA cloning kit (Invitrogen), and specificity was confirmed by DNA sequencing (data not shown). Calculation of the relative expression levels of each target was conducted based on the cycle threshold ($C_T$) method [31]. The $C_T$ for each sample was calculated using the Opticon Monitor Analysis Software (MJ Research) with an automatic fluorescence threshold ($R_n$) setting. The efficiency of the PCR was assessed for each target by the amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Depending on specific PCR conditions, efficiencies ranged from 98% to 102%. Accordingly, the fold expression over control values was calculated for each target by the equation $2^{-\Delta\Delta C_T}$, where $\Delta C_T$ is determined by subtracting the corresponding 18S $C_T$ value (internal control) from the specific $C_T$ of each target and experimental condition, and $\Delta\Delta C_T$ is obtained by subtracting the $\Delta C_T$ of E-treated groups (STE or LTE) from that of the respective control group (STC or LTC, assigned a reference value of 100). No significant differences in $C_T$ values were observed for 18S rRNA between the different experimental groups.

**Table 1**
Primers and PCR products for QPCR.

| Gene | Primer sequence (5′-3′) | Product size (bp) |
|---|---|---|
| mBDNF | Forward: ACCATAAGGACGCGGACTTG<br>Reverse: GAGGAGGCTCCAAAGGCAC | 69 |
| mBDNF I | Forward: CCTGCATCTGTTGGGGAGAC | 175 |
| mBDNF II | Forward: CTAGCCACCGGGGTGGTGTAA | 144 |
| mBDNF III | Forward: CTTCCTTGAGCCCAGTTCC | 126 |
| mBDNF VI | Forward: CAGAGCAGCTGCCTTGATGTT | 159 |
| mBDNF V | Forward: TTGGGGCAGACGAGAAAGC | 222 |
| mBDNF 5′ UTR | Reverse: GCCTTGTCCGTGGACGTTTA | |
| 18S | Forward: TAAGTCCCTGCCCTTTGTACACA<br>Reverse: GATCCGAGGGCCTCACTAAAC | 71 |

### *2.5. Immunohistochemistry*

The affinity-purified antibody for ERalpha (clone 6F-11, 1:200 dilution) was purchased from Novocastra (Newcastle upon Tyne, UK). The anti-BDNF antibody (sc-546, 1:800 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For specificity validation tests, the antigenic peptide (sc-546P, Santa Cruz Biotechnology) was used to pre-adsorb BDNF antibodies by incubating 1 μg of the antibody with 10–20 μg of the peptide for 24 h at 4 °C. For negative controls, the antibody-antigen complexes (pre-adsorbed antibodies) were applied to control

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tissues in immunohistochemical assays. The anti-SYN antibody (clone SY38, 1:2000 dilution) was purchased from Chemicon International (Millipore Corp., Billerica, MA, USA). Anti-rabbit and anti-mouse secondary antibodies (biotin conjugate, B8895/B8774, 1:200 dilution) were purchased from Sigma (Sigma, St. Louis, MO, USA).

Brain serial sections (5 μm thick) were mounted on 3-amino-propyltriethoxysilane (Sigma)-coated slides and dried for 24 h at 37 °C. All primary antibodies were incubated overnight at 4 °C. 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 (PMyR, Buenos Aires, Argentina).

Each immunohistochemical run included positive and negative controls. For negative controls, the primary antibodies were replaced with nonimmune mouse or rabbit serum (Sigma). In addition, the specificity of the antibodies was tested by Western blot analysis according to the previously described by Varayoud et al. [32].

To obtain semiquantitative data for protein expression, image analysis was performed using the Image-Pro Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as previously described [33,34]. In brief, 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), using a D-plan 10x objective (NA = 0.25). 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. Typically, eight or more sections of a one-in-six series were scored and averaged per animal. The images of immunostained slides were converted to greyscale, and the granule layers of the DG, the polymorphic zone (PZ), and the CA1 and CA3 regions of the hippocampus were delimited according to Paxinos and Watson [35]. For BDNF and SYN, the integrated optical density (IOD) was measured as a linear combination of the average grey intensity of the immunostaining and the relative area occupied by positive cells. This morphometric approach provides good reproducibility because relative spatial variables are independent of the oversampling errors that would be incurred in calculating the absolute volumes and total cell numbers [36,37]. The ERalpha (+) cell population was represented as spatial cell densities (number of cells/mm$^2$ of evaluated area calculated by means of Image Pro-Plus software). Comparisons were made from the dorsal hippocampus and the same area of the cell layers (see Fig. 3).

### 2.6. DNA extraction and bisulfite modification

DNA was isolated from microdissected hippocampi using the Wizard® Genomic DNA Purification Kit (Promega). DNA (2 μg) in a volume of 50 μl was denatured with NaOH for 20 min at 37 °C.

**Table 3**
Primers and PCR products for QMSP and COBRA.

| Primer | Primer sequence (5′-3′) | Product size (bp) |
|---|---|---|
| E5M | Forward: GTGTAAGGCGATTAGGGATATTTC<br>Reverse: ATAACTACTTAAAAAAAACGACGCG | 106 |
| E4 DMS | Forward: TAATGATAGGTTTGGTTTTTGTGTG<br>Reverse: AACTATCATATAATACCTCCTCTACCTC | 193 |
| E4CaRE | Forward: TTATAAAGTATGTAATGTTTTGGAA<br>Reverse: AATAAAAAAATAAATAAAAATCCAC | 188 |
| E4CRE | Forward: TGTGTTGTTGTTGTTTAGATAATGATAG<br>Reverse: CAAAAACTAAAAAATTTCATACTAACTC | 342 |

Thirty microliters of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5, freshly prepared, were added and mixed, and samples were incubated in mineral oil at 55 °C for 16 h [38]. Modified DNA was purified using Wizard® DNA purification resin according to the manufacturer (Promega) and eluted into 50 μl of heated water (60–70 °C). Modification was completed by NaOH treatment for 15 min at 37 °C, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20 °C. Unmethylated mouse genomic DNA (New England BioLabs, Beverly, MA, USA) was used as a negative control for methylated genes.

### 2.7. Methylation of genomic DNA

CpG islands in genomic DNA were methylated using *Sss* I methyltransferase (New England BioLabs) according to the Tost and Gut's protocol [39]. After methylase treatment, the positive control would have 100% methylated cytosines in all cytosine sites.

### 2.8. Combined bisulfite restriction analysis (COBRA)

In COBRA, restriction enzyme digestion is employed to reveal DNA methylation-dependent sequence differences in PCR-amplified bisulfite-treated genomic DNA [40]. Using the MethPrimer software, we identified CpG-rich fragments on the promoter IV of BDNF. These regions were confirmed to contain at least one restriction site for *Bst* UI, *Taq* I and/or *Mae* II enzymes to evaluate the following methylation-sensitive sites: depolarization-dependent methylation site (DMS), Ca$^{2+}$-responsive elements (CaRE) and cyclic adenosine monophosphate response element (CRE) (Fig. 5A; Table 2). PCR primers were designed to encompass these regulatory regions and to complement the bisulfite-converted DNA (Table 3). The PCR mixture contained 2 mM $MgCl_2$ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), 10 pmol of each primer (Invitrogen), and bisulfite-modified DNA (1 μl) in a final volume of 25 μl of 1X PCR buffer. Reactions were hot-started at 95 °C for 10 min before the addition of 2.5 U *Taq* DNA polymerase (Invitrogen). Amplification was carried out for 40 cycles (30 s at 96 °C, 30 s at 60 °C, and 45 min at 72 °C), followed by a final 8 min

**Table 2**
Regulatory regions in the promoter IV of BDNF evaluated by combined bisulfite restriction analysis (COBRA) procedures.

| Regulatory regions | Role | Distance to the transcription initiation site (bp) | Restriction site in the native sequence |
|---|---|---|---|
| DMS[a] | Regulates transcription depending on the neuronal activity | −111 | *Bst* U I<br>5′-GCGCGGA-3′ |
| CaRE[a] | E would produce a Ca$^{2+}$-mediated regulation of elements acting in *trans* to this regulatory sequence | −71 | *Taq* I<br>5′-CTATTTCGAG-3′ |
| CRE[a] | E would produce a cAMP-mediated regulation of elements acting in *trans* to this regulatory sequence | -37 | *Mae* II<br>5′-TCACGTCA-3′ |

[a] DMS: depolarization-dependent methylation site; CaRE: Ca$^{2+}$-responsive element and CRE: cyclic adenosine monophosphate response element.

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extension at 72 °C. Controls with unmodified DNA and without DNA were performed for each set of primers. To ensure complete digestion of the PCR products in the subsequent restriction digestion step, each PCR product (20 μl) was loaded onto 2% agarose gel containing ethidium bromide (Sigma), quickly visualized under UV illumination and purified using the Wizard© SV Gel and Clean-Up System kit according to the manufacturer's protocol (Promega). Fifteen microliters of each purified PCR product was then digested separately with *Bst* UI (recognition site *CGCG*), *Taq* I (recognition site *TCGA*) and *Mae* II (recognition site *ACGT*). Cleavage occurs only if the CG sequence has been retained during the bisulfite conversion by a methylated cytosine residue. Then, the digested fragments were subjected to electrophoresis on 3% agarose gels. In the mixed population of resulting PCR fragments, the ratio of band intensity of digested fraction to the combined intensities of both digested and non-digested fractions reflected the levels of DNA methylation on the restriction sites [41]. Band intensity was calculated using the image analyzer Image-Pro Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MA, USA). To ensure that the bisulfite conversion was complete, a control digest was performed with *Mse* I (recognition site *TTAA*, data not shown). The molecular sizes of PCR and restriction products were determined by comparison with DNA standard size markers (Cien Marker, Biodynamics, Buenos Aires, Argentina).

### 2.9. Real-time quantitative methylation-specific PCR (QMSP)

Using MethPrimer software (http://www.urogene.org/methprimer/index1.html) [42], we identified a CpG-rich sequence in the region upstream of exon V of the mouse *BDNF* gene. This CpG island was recognized in the region corresponding to −140 and −80 bp, considering +1 the transcription start site (accession number AY057907) (Fig. 5C). After sodium bisulfite conversion, genomic DNA was analyzed using the Engine Opticon System (Bio-Rad) and SYBR Green I dye (Cambrex) as described previously, with some modifications [43]. Briefly, two sets of primers designed specifically for bisulfite-converted DNA, were used: (a) one primer set (E5 M) that anneals to the methylated sequence of the BDNF promoter V and (b) a reference set that lacks any CpG dinucleotides (primers used for the study of DMS regulatory region in BDNF promoter IV, E4 DMS) (Table 3). This set allows for equal amplification regardless of the methylation state of the bisulfite-converted DNA and provides an index of the level of modified DNA in each sample (sample normalization). The PCR mixture contained 4 mM $MgCl_2$ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), 20 pmol of each primer (Invitrogen) and bisulfite-modified DNA (1 μl) in a final volume of 25 μl of 1X SYBR Green PCR buffer. Reactions were hot-started at 95 °C for 5 min before the addition of 2.5 U *Taq* DNA polymerase (Invitrogen). Amplification was carried out for 35 cycles (15 s at 95 °C, 15 s at 57 °C, 15 s at 72 °C). Specifically for QMSP, the fluorescence measurements were performed at 75 °C because of the low melting temperature of the PCR products. The reactions were run in triplicate for each individual sample. The specificity of the reactions for methylated DNA was confirmed separately using unmethylated mouse genomic DNA and *Sss* I-treated genomic mouse DNA (in vitro methylated DNA). No amplification products were obtained when the unmethylated DNA was amplified with the E5 M primer set. Standard curves were generated by amplifying (with the E5 M set of primers) serial dilutions of the in vitro methylated DNA with increasing quantities of unmethylated DNA (the final concentration of the total DNA – methylated + unmethylated – was exactly the same in each reaction tube). The calibration curves were used to quantify the relative degree of methylation of each experimental DNA sample expressed as the ratio of the level of methylated DNA to the level of modified DNA from the quantification with E4 DMS primers.

### 2.10. Data analysis

All data are expressed as the means ± S.E.M. The effects of E treatment were analyzed with the Kruskal–Wallis test followed by Dunn's post-test. All differences were considered significant at $p < 0.05$.

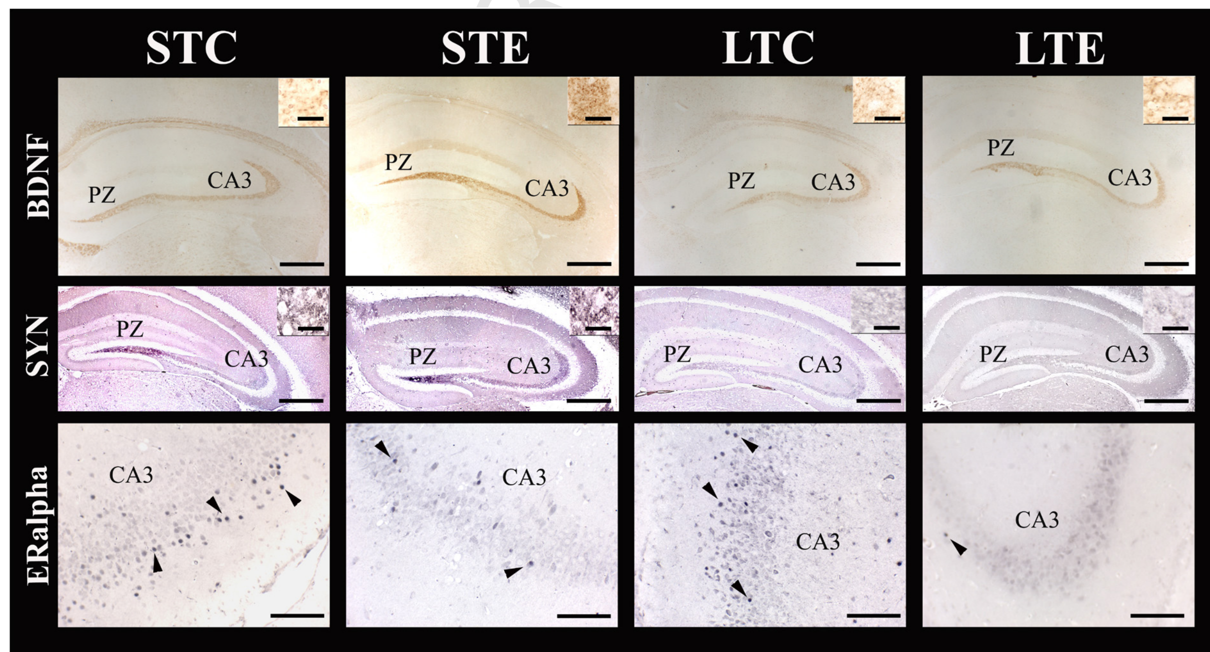


**Fig. 2.** Representative photomicrographs of immunohistochemical detection of BDNF, SYN and ERalpha in the hippocampi of ST and LT animals. Positive ERalpha nuclear staining is indicated by black arrows. BDNF and SYN, scale bar 1.000 μm (insets, scale bar 50 μm); ERalpha, scale bar 100 μm.

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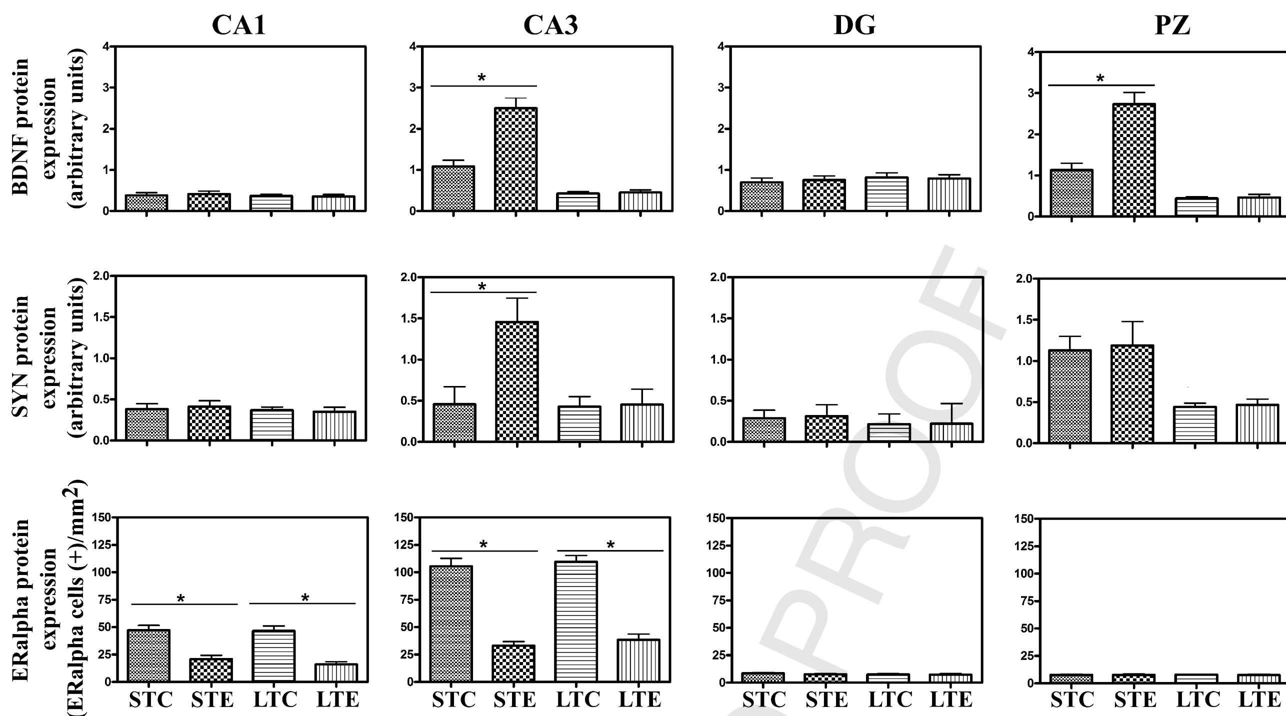


**Fig. 3.** Immunohistochemical quantification of BDNF, ERalpha and SYN in different regions of the hippocampus. Significant increases in BDNF and SYN expression were observed in the CA3 region of STE animals, while in the PZ of ST animals, a significant increase in SYN expression was observed regardless of treatment. E treatment produced a significant down-regulation of ERalpha in the CA3 and CA1 regions. Bars represent mean values (±SEM) of 6 animals per group (*, $p < 0.01$).

## 3. Results

### 3.1. Serum levels

First we measured the serum levels of E in those animals that were OVX and were not treated with E (STC and LTC groups). The levels of E in these animals were low and not shown differences between these control groups (E serum levels expressed in pg/ml: STC: $4.0 \pm 2.1$ vs. LTC: $2.1 \pm 1.3$, $p > 0.05$). In addition we measured E serum levels 4 h after E injection. No differences in serum concentrations were found between STE and LTE animals (STC: $65 \pm 2.8$ vs. LTC: $60 \pm 3.2$, $p > 0.05$).

### 3.2. The post-OVX timeframe elapsed before HRT initiation is critical for the estradiol-mediated induction of neurotrophic proteins

The expression of BDNF, SYN and ERalpha was determined in neurons from the hippocampal formation using an immunohistochemical approach (Fig. 2). BDNF immunostaining was observed in the soma and the axonal extensions of neurons located in the CA1, CA3, PZ, and DG regions. After HRT initiation, the animals treated with a short period of castration (STE) presented a higher expression of BDNF compared with controls (STC) (Fig. 3, $p < 0.01$). Increased BDNF was observed in the CA3 and PZ subfields of hippocampus. When HRT was administered 7.5 months post-OVX, E did not induce BDNF protein (LTC vs. LTE; Fig. 3).

To determine whether the timeframe elapsed before HRT initiation could affect synaptic plasticity, we studied the expression of SYN in our experimental model. The animals that underwent a short period of castration (STE) showed a significant increase in SYN expression in the CA3 region (Fig. 3, $p < 0.01$). In contrast, E did not induce SYN expression when treatment was initiated at 7.5 months post-OVX (Fig. 3).

The hippocampal regions that showed the highest immunostaining for ERalpha were CA3 and CA1. In PZ and DG, ERalpha (+) cells were observed only in a dispersed fashion. The E treatment resulted in a down-regulation of ERalpha in both STE and LTE animals (Fig. 3).

### 3.3. Effects on hippocampal BDNF mRNA levels and their splice variants

The quantification of *BDNF* mRNA levels was performed by QPCR using oligonucleotides that hybridize in the coding region of exon VI (common to all variants of alternative splicing). In parallel to the protein changes, we found a 2.5-fold increase in *BDNF* mRNA expression in the STE group (Fig. 4A, $p < 0.05$ vs. STC). In contrast, there were no significant differences in expression of *BDNF* mRNA in the LT groups.

A detailed mRNA analysis of the hippocampus revealed changes in the relative abundances of three BDNF transcripts (Fig. 4B, Table 4). We found that the upregulation in total *BDNF* mRNA levels (observed in the STE group) was mediated by means of the increased expression of variants IV and V (EIV-BDNF and EV-BDNF ($p < 0.01$). In addition, variant II (EII-BDNF) showed a small but significant increase in the STE group ($p < 0.01$ vs. STC). In the LTE group, only a mild increase in EII-BDNF variant was detected ($p < 0.01$ vs. LTC), without changes in the other splice variants.

### 3.4. The loss of E responsiveness is associated with an increase in the methylation pattern of BDNF regulatory regions

As BDNF was not induced in LTE animals, we investigated the regulatory mechanism that might underlie this lack of E response. We hypothesized that the methylation level of regulatory regions in BDNF promoters IV and V could be affected in LT groups. Based on the promoter sequence, we used two techniques for assaying

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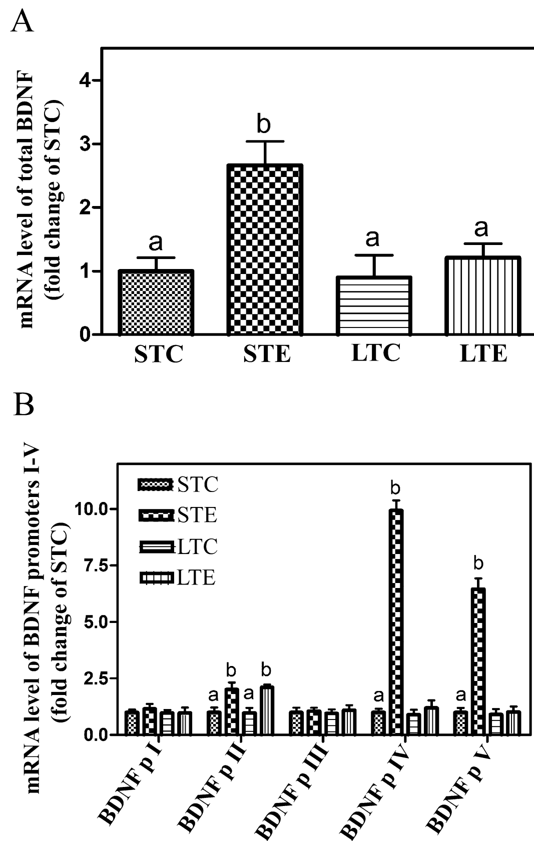


**Fig. 4.** Effects of time of castration on the effectiveness of HRT to increase the expression of BDNF mRNA. Expression levels of total BDNF mRNA (A) or its splice variants and (B) in the OVX female mice hippocampus. Control values were assigned to a reference level of 100. Different letters indicate significant differences between experimental groups ($p < 0.05$ vs. control), and values are given as means ± SEM ($n = 5$ mice/group).

**Table 4**
Relative abundances of the different BDNF transcripts relative to variant BDNF p I in each experimental group. Expression level of variant BDNF p I was assigned to a reference level of 100.

| | STC | STE | LTC | LTE |
|---|---|---|---|---|
| BDNF p I | 100.00 | 100.00 | 100.00 | 100.00 |
| BDNF p II | 114.00 | 174.78 | 110.00 | 215.31 |
| BDNF p III | 94.00 | 92.17 | 98.00 | 111.22 |
| BDNF p IV | 102.00 | 863.48 | 104.00 | 122.45 |
| BDNF p V | 98.00 | 560.87 | 101.00 | 104.08 |

DNA methylation. COBRA assay was not performed in the BDNF promoter V because its sequence has not restriction sites to *Bst* UI, *Taq* I, and/or *Mae* II. On the other hand, QMSP was not applied to study BDNF promoter IV because all the primers pairs have inacceptable thermodynamic quality to perform quantitative PCR reactions.

The COBRA analysis showed that *Sss* I-treated genomic mouse DNA, used as a positive control (M), was completely digested by restriction enzymes, whereas non-methylated DNA used as a negative control (U) remained unchanged (Fig. 5B). Two regulatory regions of promoter IV, the DMS and CaRE sites, were completely demethylated in all experimental groups, demonstrated by the lack of DNA digestion with the respective enzymes. In contrast, the CRE site of promoter IV showed a high level of methylation (75 ± 4%, $p < 0.05$) in both LT groups (LTC and LTE). The ST groups (STC and STE) presented completely demethylated CRE sites (Fig. 5B).

Analysis within promoter V of BDNF was performed by QMSP. The results revealed that the CpG island had a higher level of methylation in the LT groups (both LTC and LTE) relative to the ST groups ($p < 0.05$, Fig. 5D). These data indicate an overall increase in the methylation of CG regions of both promoters that occurs in the hippocampi of long-term OVX animals.

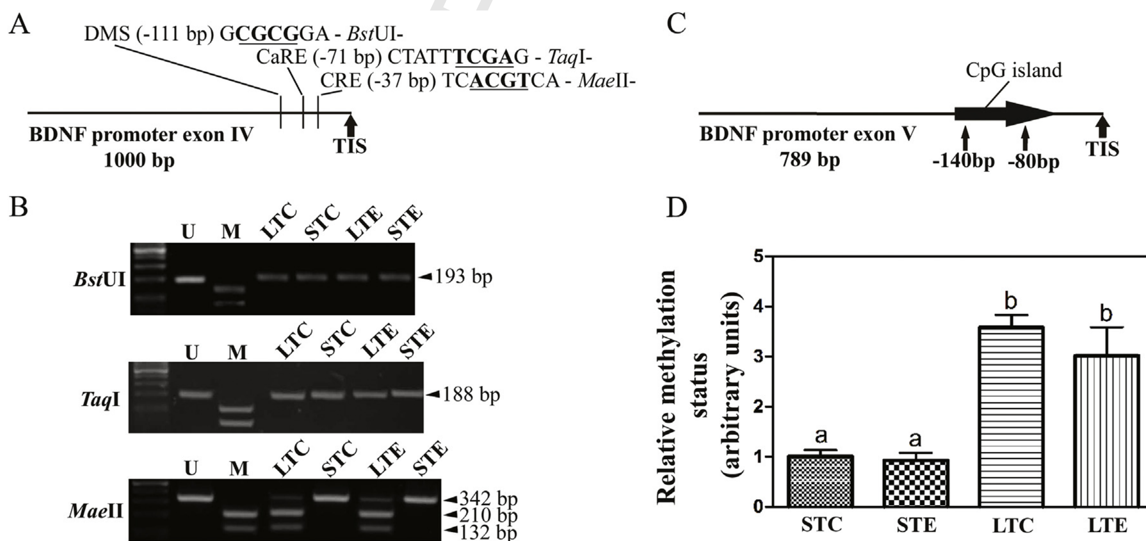


**Fig. 5.** Methylation pattern analysis of *BDNF* gene regulatory regions. (A) Genomic organization of regulatory sites on BDNF promoter IV. DMS, depolarization-dependent methylation site; CaRE, $Ca^{2+}$-responsive elements; CRE, cyclic adenosine monophosphate response element; TIS, transcription-initiation site. (B) Representative ethidium bromide-stained gel electrophoresis of COBRA assay with the restriction enzymes *Bst* UI, *Taq* I and *Mae* II. U, non-methylated genomic mouse DNA used as a negative control; M, *Sss* I-treated genomic mouse DNA, used as a positive control. (C) CpG dinucleotides position on the CpG island analyzed in the BDNF promoter V. TIS, transcription-initiation site. (D) Quantitative evaluation of BDNF promoter V methylation by QMSP. The results are expressed as relative levels of methylation. STC values were assigned to a reference level of 1, and values are given as means ± SEM. Different letters indicate significant differences ($p < 0.05$ vs. STC, $n = 5$ mice/group).

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## 4. Discussion

In menopause, the ovarian steroid declination may influence cognitive abilities of older women, and there is evidence that HRTs with E mimetics may provide benefits in some, but not all, women. It may be that the likelihood for positive responses is related to the timing of E replacement following E decline [44]. In the present work, we show that the post-OVX timeframe elapsed before HRT initiation is critical for the E-mediated induction of neurotrophic proteins such as BDNF and SYN. The animal model used in our work did not represent a physiological menopause, however it was very useful to study the loss of E responsiveness of the *BDNF* gene observed in the hippocampus of long-term OVX animals and the association of this event with an increment in the methylation status of specific *cis*-acting elements located in promoters IV and V.

Evidence from basic research in rodents indicates that the responsiveness of the mammalian nervous system to estrogen is altered after a period of ovarian hormone deprivation [45]. The results of several reproductive behavior studies revealed a decreased ability of estradiol to induce sexual behavior in ovariectomized rats if the estradiol is administered after prolonged hormone deprivation [46,47]. In addition, estradiol treatment initiated within 3 months, but not 10 months, after ovariectomy enhanced the acquisition of a T-maze spatial memory task in aged rats, suggesting a window of opportunity after the loss of ovarian function during which hormone replacement can effectively prevent the effects of aging and hormone deprivation on cognitive function [29]. Women's Health Initiative Memory Study (WHIMS), the largest randomized, controlled trial of hormone therapy ever undertaken, not only failed to detect a beneficial effect of estrogen or estrogen plus progestin therapy on cognitive aging but also found that such therapies actually caused harm (cognitive decline and dementia) in women who were aged an average of 72 years at the time of the initiation of therapy [48].

The length of the estradiol treatment, the post-OVX interval, and the age of estradiol replacement initiation [49] are all important factors in the regulation of specific cellular systems, but the molecular mechanisms associated with these events are not fully elucidated. Our study evaluated the hypothesis that post-OVX timeframe elapsed before HRT initiation is critical for the E-induction of neurotrophic proteins in the mouse hippocampus. In this context, we found that estradiol treatment significantly increased either *BDNF* mRNA and protein levels in the hippocampus only if the post-OVX timeframe elapsed before HRT initiation is short (15 days in STE animals vs. 7.5 months in LTE animals). Several studies have examined the effect of estradiol on *BDNF* mRNA expression in female rats. Chronic estrogen deprivation reduced *BDNF* mRNA levels in several cortical and hippocampal regions [18,22]. *BDNF* mRNA levels in some of the hippocampal fields could be restored over several weeks (5–25 weeks) if the estrogen replacement was initiated relatively rapidly after the OVX (within 3 weeks) but not following a long-term estrogen deprivation [22]. Consistent with these observations, there is evidence that the post-OVX interval is an important factor for the regulation of some genes, such as *N*-methyl-D-aspartic acid (NMDA) receptor mRNA levels [50] or muscarinic acetylcholine receptors (mAChRs) and acetylcholinesterase (AChE) activity in the hippocampus [51]. In addition, BDNF protein levels were studied instead of mRNA, because *BDNF* mRNA does not always predict protein expression [52,53]. We found that if the post-OVX timeframe elapsed before HRT initiation is short, E treatment can increase BDNF protein levels. These changes were evident in the CA3 and PZ subfields of hippocampus, where BDNF normally is anterogradely transported to the axons (mossy fibers) from their primary source, the granule cells [54,55].

QPCR analysis allowed us to determine that the increased expression of *BDNF* in the STE group was largely due to the induction of the EIV- and EV-BDNF variants and, to a lesser extent, EII-BDNF. In the LTE group, only a slight increase in BDNF variant II was observed. Apparently, the combination of alternative transcriptional initiation sites provides a variety of BDNF transcripts that seem likely to differ in their stability and/or in the intracellular compartments into which they are trafficked and translated [56].

Previous results showed that BDNF expression may be regulated by epigenetic modifications, such as a higher DNA methylation of regulatory regions triggering its silencing [57]. An increase in the DNA methylation of *BDNF* promoter IV and a consequent decrease in *BDNF* mRNA in the rat prefrontal cortex was found in association with exposure to periods of abusive maternal care (dragging, rough handling) [58]. These findings establish a relationship between epigenetic modifications and long-term regulation of *BDNF* expression. In fact, there is evidence suggesting that certain processes involving the remodeling of chromatin in promoter regions of BDNF regulate the expression of transcriptional variants [59]. The differences that we found in the expression levels of BDNF transcription variants led us to ask whether the lack of response to treatment with E observed in LTE animals is due to epigenetic changes that would affect the activity of BDNF promoters IV and V. We postulated that the silencing could be due to a process of differential methylation in *cis*-elements of the promoters.

COBRA showed that the DMS and CaRE regulatory regions of BDNF promoter IV are not methylated in the four experimental groups, while the CRE regulatory region showed a higher number of methylated alleles in the animals that underwent a long period of castration. E has the ability to promote cAMP accumulation in hypothalamic neurons [60] and human neuroblastoma cells [61]. This accumulation generates an increase in phosphorylation of CREB and transcriptional activation of genes that have cAMP response elements. Thus, there is the possibility that CREB represents a convergence of multiple signal transduction events mediated by E [62]. Taken together, these results could indicate that one way in which the time of castration could affect E induction of BDNF expression would be through differential methylation of regulatory regions, particularly in the CRE region. The QMSP results for promoter V showed that LT animals had a higher level of methylation in the CpG island compared to the ST group. These results and the COBRA results would indicate that the failure of estrogen induction of BDNF in the chronically castrated animals is associated with transcriptional silencing of the promoters IV and V of the *BDNF* as a consequence of a higher level of methylation.

Changes in the methylation status of BDNF observed in the LT animals may be also linked to modifications in the expression of DNA methyltransferases (DNMTs). In a previous work it has been shown that DNMTs activity is modified with age, increasing from young to old [63]. In these context, changes in DNMTs expression and/or activity in LT animals could not be ruled out.

Additionally, our results suggest that E does not change methylation status of *BDNF* gene. So, how does E increase BDNF expression in the short term ovariectomized animals? Estrogen-mediated regulation of BDNF could involve several mechanisms besides direct regulation of *BDNF* gene expression involving nuclear ERalpha and/or ERbeta. Among them we can highlight the indirect effects mediated via estrogen action on GABAergic interneurons [64]. This line of reasoning is supported by the demonstration that ERs are localized in GABAergic neurons throughout hippocampus [65], that estrogen appears to depress inhibition in the hippocampus [66] and that BDNF synthesis is activity-dependent [67]. Thus, estrogen-mediated inhibition of GABAergic activity could secondarily increase activity in the granule cells, thereby indirectly increasing BDNF synthesis.

On the other hand, there may be an ovarian factor other than E that could explain the changes in the methylation status of BDNF

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observed in the LT animals. In the study by Gibbs [15], changes in the expression of *BDNF* mRNA in the dentate gyrus and CA1 were larger in the afternoon of proestrus, when hormones other than E (particularly progesterone) could also affect hippocampal function. In this sense, the effects of progestins, often given in combination with estrogens in hormone treatment regimens, on cognition across the life span are not well understood. Even less is known as to how co-administration of progestins with estrogens will impact the critical window of estrogen effects on cognition [4].

The HRT significantly increased the protein levels of SYN in STE animals. In contrast, E did not induce SYN expression when treatment was initiated at 7.5 months post-OVX (LTE). SYN belongs to the presynaptic vesicle proteins that play an important role in synaptic plasticity and cognitive function [68]. The loss of SYN expression in the hippocampus has been correlated with cognitive decline in Alzheimer's disease [69]. On the contrary, it is suspected that increased expression of synaptic proteins may be partially responsible for the improved learning and memory performance following HRT in E treated-OVX mice [70]. In accordance with the BDNF response, SYN-induction is affected when E treatment begins after a long period of castration.

One mechanism by which E may enhance BDNF expression in the hippocampus is nuclear ERalpha induction of BDNF expression [71]. Taking this possibility into account, we determined the ERalpha expression in the different experimental groups and detected a downregulation of ERalpha in response to E in both short and long term OVX animals. In our model, the differences found in the BDNF response to E are unlikely to be a result of a change in the ERalpha expression in hippocampal neurons.

In conclusion, the results of the present study support the hypothesis that long-term ovarian hormone deprivation negatively affects the E induction of neurotrophic proteins. We propose that an increase in *BDNF* methylation could explain the lower estrogenic response after a long ovarian hormone deprivation. Because the time point of OVX is different between STE and LTE animals, we could not rule out the possibility that the preserved ovarian functionality in STE animals for a long term could be responsible of the E responsiveness observed in this group. Further studies examining other epigenetic pathways in the *BDNF*, such as the regulation of histone acetylation, will contribute to better understanding of the mechanism for E regulation of BDNF expression.

## Conflict of interest

Q2 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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