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Epigenetic regulation of estrogen receptor α contributes to age-related differences in transcription across the hippocampal regions CA1 and CA3

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Abstract

The expression of estrogen receptor alpha (ER α) varies across brain regions and changes with age and according to the previous history of estradiol exposure. ER α is regulated by a number of mechanisms including the level of mRNA (*Esr1*) expression. For this study, we took advantage of regional differences in hippocampal ER α expression to investigate DNA ER α promoter methylation at CpG dinucleotide sites as a potential epigenetic mechanism for regulating gene expression. Young and aged female Fischer 344 rats were ovariectomized and *Esr1* expression and ER α promoter methylation were examined in hippocampal regions CA1 and CA3, either 3 or 14 weeks following surgery. The results indicate that reduced *Esr1* expression in region CA1 relative to CA3 was associated with an increase in DNA methylation in region CA1, particularly for the first CpG site. Additionally, differential methylation of distal CpG sites, 11–17, was associated with altered *Esr1* expression during aging or following long-term hormone deprivation. The results support the idea that methylation of site 1 may be the primary regulatory region for cross-regional patterns in ER α expression, while distal sites are modifiable across the life span and may act as a feedback mechanism for ER α activity.

Keywords

Aging; estrogen receptor alpha; hippocampus; transcription; DNA methylation

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Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. No human Subjects were used in the current study.

All authors have reviewed and approved the manuscript.

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1. Introduction

Estradiol (E2) influences several biological processes that likely contribute to neuroprotection (Bean, Ianov et al. 2014). The relative levels and subcellular distributions of ER α varies across brain regions and according to the previous history of E2 exposure (Milner, McEwen et al. 2001, Mitra, Hoskin et al. 2003, Mehra, Sharma et al. 2005, Mitterling, Spencer et al. 2010). Differences in ER α expression/activity likely contribute to regional differences in vulnerability to ischemia and oxidative stress (Merchenthaler, Dellovade et al. 2003, Zhang, Raz et al. 2009). While the expression profile for ER α in the hippocampus is well characterized by age and hippocampal subregions, the molecular mechanisms that regulate estrogen receptor expression in the hippocampus are not well understood (Bean, Ianov et al. 2014).

One mechanism for the regulation of gene expression is through methylation of cytosines in guanine-cytosine rich areas of the gene promoter region, termed CpG islands. In several tissues, ER α promoter methylation increases with age and is associated with decreased ER α expression and increased incidence of disease (Post, Goldschmidt-Clermont et al. 1999, Li, Shiina et al. 2004). Similarly, in the brain, ER α promoter methylation is associated with a decrease in ER α expression and underlies physiological and behavioral differences across the lifespan (Schwarz, Nugent et al. 2010, Gore, Walker et al. 2011). We examined the DNA methylation status of the 17 CpG sites within the ER α promoter exon 1b region in ovariectomized female rats to test the hypothesis that CpG DNA methylation is an active epigenetic regulator of regional and age-related differences in the expression of ER α mRNA, *Esr1*. For this study, we took advantage of regional differences in hippocampal ER α expression, with increased expression in region CA3 relative to region CA1 (Rune, Wehrenberg et al. 2002, Mehra, Sharma et al. 2005), and possible auto-regulation of ER α promoter activity by E2 (Castles, Oesterreich et al. 1997, Donaghue, Westley et al. 1999, Pinzone, Stevenson et al. 2004), which may underlie effects of hormone deprivation on ER α expression (Bean, Ianov et al. 2014). The results suggest that differential methylation of sites within the ER α promoter may regulate transcription of *Esr1* across hippocampal regions and that DNA methylation of distal CpG sites may have a function in age-related expression changes relative to upstream sites in the promoter.

2. Materials and Methods

2.1. Animals

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Female Fischer 344 rats of two ages, young (3 months, n = 21) and aged (18 months, n = 22) were obtained from National Institute on Aging colony at Charles River Laboratories, through the University of Florida Animal Care and Service facility. Animals were maintained on a 12:12 hour light schedule, and provided *ad lib* access to food and water.

2.2. Surgery and tissue collection

Ovariectomy (OVX) was performed as previously described (Sharrow, Kumar et al. 2002, Bean, Kumar et al. 2015). Briefly, rats were handled for 5 min a day for at least 1 week prior to surgery. Female rats were ovariectomized under isoflurane (Piramal Healthcare) in oxygen using a VetEquip anesthesia system. Bilateral incisions were made to expose the ovaries, which were cleared from the fat tissue and dissected out. Subsequent to the closure of the incisions, buprenorphine (0.03 mg/kg) and saline (5–10 ml) were given by subcutaneous injection. Following OVX, the food of the animals was exchanged to a casein-based chow, which contains lower levels of phytoestrogens. Three weeks (wk) following OVX (young, n=10 and aged, n=11), rats were overdosed with CO₂, decapitated, and the hippocampi were dissected. The same procedure was repeated for the remaining animals after a 14 week period (young, n=11 and aged, n=11) (Fig 1). Hippocampal regions (CA1 and CA3) were separated, placed in tubes, immediately frozen in liquid nitrogen, and stored in –80°C until processed.

2.3. RNA isolation and reverse transcription quantitative polymerase chain reaction

RNA was isolated from each hippocampal region (n=5–6 per region of each age and OVX duration group) using the RNeasy Lipid Tissue Mini kit (Qiagen, catalog number: 74804) and DNase digestion was performed with the RNase-Free DNase Set (Qiagen, catalog number: 79254). Following isolation, the concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, catalog number: 205311) and quantitative polymerase chain reaction (qPCR) was completed using the TaqMan Gene Expression Assays (*Esr1*: Rn01640372_m1, *Gapdh*: Rn01775763_g1) in a 7300 Real-Time PCR system with SDS software version 1.3.1 (Applied Biosystems). The CT method (Livak and Schmittgen 2001) was used to determine the relative cDNA levels and the CA1 region from young short-term rats were used as the calibrator samples.

2.4. Sodium bisulfite sequencing

Genomic DNA was isolated from the CA1 and CA3 areas (n=5 per age group and OVX time) using the DNeasy Blood & Tissue kit (Qiagen, catalog number: 69504). The DNA concentration was quantified using the NanoDrop 2000 spectrophotometer and sodium bisulfite conversion was performed with the EZ DNA Methylation-Gold kit (Zymo Research, catalog number: D5005) according to the manufacturer's directions. The exon 1b promoter region of ER α (GenBank accession number: X98236) was amplified with the following modifications from previous reports (Champagne, Weaver et al. 2006, Kurian, Olesen et al. 2010). The thermocycler parameters included an initial denaturation cycle of 5 minutes at 94°C, 40 cycles of 1 minute at 94°C (denaturing), 3 minutes at 56°C (annealing) and 1 minute at 72°C (extension), followed by a final extension cycle of 15 minutes at 72°C. The following bisulfite sequencing PCR (BSP) primers were used: outer forward 5'TAGTATATTTTGATTGTTATTTTAT3'; outer reverse 5'CTAAACAAAAAATAAATTACTTTTC3'. The PCR product was used for nested PCR with the same thermocycler parameters with the following BSP nested primers: forward 5'TTTATTTGTGGTTTATAGATATTT3' and reverse

5'ACAAAAAAAAAAAAATCAAAACAC3'. In addition, a preliminary check of bisulfite conversion efficiency of each sample was assessed by performing a separate nested PCR reaction. The same thermocycler conditions were used with wild type sequence-specific primers, which map to the unconverted DNA sequence of the exon 1b promoter region of ER α (outer forward 5'CAGCACACTTTGACTGCCATTCTAC3'; outer reverse 5'CTAGGCAGAAAGGTAAGTTGCTTTC3'; nested forward 5'TTTATCTGTGGTTTACAGACATCT3'; nested reverse 5'ACAGAAAGAGGGAAATCAAAACAC3'). Amplification of the target sequence with wild-type primers would indicate incomplete bisulfite conversion. All samples demonstrated complete bisulfite conversion. Unconverted DNA was used as the positive control for the wild-type primers.

The nested product (459bp) of each PCR reaction using BSP primers was cloned with the TOPO TA cloning kit for Sequencing (Life Technologies, catalog number: K4575-J10) with the following modifications to the transformation reaction: TOP10 cells were heat-shocked for 45 seconds at 42°C and immediately transferred to ice for 7 minutes before the addition of S.O.C. medium. Positive clones were confirmed by colony PCR using nested BSP primers, and miniprep was performed on each positive clone (PureLinkquick plasmid DNA miniprep, Life Technologies, catalog number: K2100-10). The samples were sent for Sanger sequencing at the Interdisciplinary Center for Biotechnology Research, University of Florida. The DNA methylation status of all 17 CpG sites from each region were analyzed using BiQ analyzer (Bock, Reither et al. 2005) retaining the default parameters. All positive clones contained conversion rates from 97–100%, and FASTA files which contained a gap in more than one CpG site were removed. After quality filtering, the average number of clones per animal/region was 10 ($\pm$ SEM 2) and the average number of clones per age and OVX groups was 51 ($\pm$ SEM 4). In addition, the total number of clones for each hippocampal region was 204 for CA1 and 203 for CA3. Hierarchical clustering and heatmap figures were generated in Partek Genomics Suite 6.6 (Partek Inc.) using clones which contained at least one site methylated to illustrate the DNA methylation pattern in site 1 to sites 2–17.

2.5. Statistical Analysis

All statistical analyses were performed using StatView 5.0 (SAS Institute Inc, NC). Analyses of variance (ANOVAs) were used to determine significant main effects for RNA expression. For CpG methylation, repeated measures ANOVAs were employed to determine main effects of age and hippocampal region across CpG sites. Fisher's protected least significant difference (PLSD) *post hoc* comparisons with $p < 0.05$ were employed to localize differences related to differential expression across CpG sites. For interactions of CpG sites with age, OVX duration, or hippocampal region, *post hoc* ANOVAs were employed to localize differences within each site. A chi-square analysis was employed to examine the independence of methylation across CpG sites.

3. Results

3.1. Region and aging effects on ER α mRNA expression

Figure 2 illustrates the expression of *Esr1* associated with age (young: 3 months, aged: 18 months), OVX duration (short-term: 3 wk, long-term: 14 wk), and region (CA1 or CA3). An increase in *Esr1* expression was observed in older animals (Fig. 2A). Similarly, expression was increased for the long-term OVX relative to short-term OVX (Fig. 2B). Finally, the largest difference was observed as a 3 fold increase in *Esr1* expression in CA3 relative to CA1 (Fig. 2C). A three factor ANOVA for expression of *Esr1* confirmed significant main effects of age [$F(1,38) = 33.14$, $p < 0.001$], OVX duration [$F(1,38) = 56.0$, $p < 0.0001$], and region [$F(1,38) = 155.42$, $p < 0.0001$]. Additionally, there was an interaction of OVX duration and age [$F(1,38) = 13.31$, $p < 0.001$], region and age [$F(1,38) = 9.25$, $p < 0.01$], and a tendency ($p = 0.063$) for a region by OVX duration interaction.

The interaction of age and OVX duration was due to increased *Esr1* expression limited to the short duration OVX (Fig. 2D). To examine the interaction of age and OVX duration, *post hoc* ANOVAs were conducted within respective OVX durations and revealed an age difference for short-term OVX [$F(1,18) = 40.27$, $p < 0.001$], with an age by region interaction [$F(1,18) = 9.52$, $p < 0.01$]. Examination of each region indicated that, for short-term OVX, an age-related increase in *Esr1* expression was observed in region CA1 [$F(1,9) = 27.6$, $p < 0.001$] and in region CA3 [$F(1,9) = 24.6$, $p < 0.0001$] (Fig. 2D).

Long-term OVX was associated with an increase in *Esr1* expression in CA1 and CA3 for younger animals. For older animals, long-term OVX increased expression only in region CA1 (Fig. 2D). To localize effects of E2 deprivation, effects of OVX duration within each region and each age group were examined. The results indicated that relative to young short-term OVX, *Esr1* expression increased in CA1 [$F(1,9) = 26.68$, $p < 0.001$] and CA3 [$F(1,9) = 60.6$, $p < 0.0001$] of young long-term OVX animals (Fig. 2D). For aged animals, long-term OVX increased *Esr1* expression [$F(1,10) = 10.45$, $p < 0.01$] in CA1 relative to aged short-term OVX rats (Fig. 2D). Thus, it appears that *Esr1* expression is increased due to age and long-term E2 deprivation. We confirmed this by comparing young short-term OVX relative to aged long-term in CA1 [$F(1,9) = 100.83$, $p < 0.0001$] and in CA3 [$F(1,9) = 75.80$, $p < 0.0001$] (Fig. 2D).

3.2. Methylation of the ER α promoter

Considerable variability in methylation was observed across the 17 CpG sites of the ER α promoter region. In general, the pattern of methylation was similar across the two regions with the greatest methylation observed at site 1 and minimal methylation for sites 2–10. Modest methylation was observed for distal sites 11–17 (Fig 3A). A repeated measures ANOVA was conducted across the 17 DNA CpG methylation sites examining main effects of region, age, and OVX duration. As expected, a significant difference in methylation was observed across CpG sites [$F(16,512) = 26.91$, $p < 0.0001$] indicative of the considerable variability in CpG methylation. *Post hoc* tests across all sites, collapsed across age, region and OVX duration, indicated the first CpG site exhibited methylation that was significantly greater than all other sites. In addition, considerable methylation was observed for sites 11,

15, and 17, which were greater than sites 2–7, 9, and 14 (Table 1). No significance difference was observed for any of the main effects; however, there was an interaction of CpG site and region [$F(16,512) = 2.49$, $p < 0.005$] and a CpG site by age by region interaction [$F(16,512) = 2.13$, $p < 0.01$].

ANOVAs were conducted within each site to examine the site by region interaction. Increased methylation was observed in region CA1 for site 1 [$F(1,38) = 4.30$, $p < 0.05$] and methylation was increased in region CA3 for site 14 [$F(1,38) = 10.34$, $p < 0.005$], site 15 [$F(1,38) = 4.12$, $p < 0.05$] (Fig. 3A). Due to the higher DNA methylation ratio on site 1 relative to downstream sites, a closer examination of the clones that contained at least one site methylated in the promoter was performed by chi-square analysis between DNA methylation in site 1 to sites 2–17.

For region CA1, chi-square analysis showed no significant associations between site 1 methylation and distal CpG methylation on sites 2–17, suggesting that methylation of these sites is independent of methylation of the first site in the exon 1b promoter (Fig. 3B). For region CA3, site 1 methylation was independent from the other sites with the exception of a significant association to DNA methylation in site 15. The analysis showed that when site 1 is not methylated, there is a higher probability of methylation on site 15 ($\chi^2 = 4.662$, $p < 0.05$).

To examine the site by age by region interaction, ANOVAs were conducted within each site and region to examine age effects (Fig. 4A–B). A significant age-related increase in methylation was observed for site 11 [$F(1,18) = 6.49$, $p < 0.05$] and 12 [$F(1,18) = 6.09$, $p < 0.05$] in CA3. In contrast, young animals exhibited a significant increase in methylation at site 15 [$F(1,18) = 4.76$, $p < 0.05$] in CA1 and site 17 [$F(1,18) = 5.57$, $p < 0.05$] in CA3.

Because *Esr1* expression was increased in area CA1 and CA3 of young animals by long-duration OVX, age differences in DNA methylation may have been masked by effects of long-term OVX in young. Therefore, we separated young animals according to OVX duration and for sites 11–17, we compared young short-term OVX, young long-term OVX, and all aged animals. For region CA1 a significant group difference was observed for site 11 [$F(2,17) = 3.96$, $p < 0.05$] and site 14 [$F(2,17) = 4.24$, $p < 0.05$]. *Post hoc* tests indicated that in each case, methylation was increased in young short-term, relative to young long-term and aged animals (Fig 4C–D). For region CA3 a significant group difference was observed for site 13 [$F(2,17) = 3.80$, $p < 0.05$] and *post hoc* tests indicated that methylation was decreased in young short-term OVX, relative to young long-term OVX (Fig 4E).

4. Discussion

Previous research using rat models indicate region and age-related changes in ER α protein expression within the hippocampus, with elevated expression in region CA3 relative to CA1 and altered expression in both regions with age or E2 deprivation (Mehra, Sharma et al. 2005, Bohacek and Daniel 2009, Zhang, Han et al. 2011). While the effect of age on *Esr1* expression is unclear (Tohgi, Utsugisawa et al. 1995, Ishunina and Swaab 2007), *Esr1* messenger levels increase following E2 deprivation (Sarvari, Kallo et al. 2014), and are

higher in area CA3 relative to CA1 (Rune, Wehrenberg et al. 2002). In the current study, we confirmed that *Esr1* expression is elevated in region CA3 relative to region CA1, suggesting that transcriptional regulation contributes to differential expression of receptor across the two regions. The age difference in *Esr1* expression was limited to the short-term OVX animals, with increased expression in all aged animals. Furthermore, expression in young animals was elevated in region CA1 following long-term hormone deprivation, such that expression was similar to that of aged animals. These results are consistent with work indicating that hormone state can regulate *Esr1* expression and indicate that long-term E2 deprivation may up regulate *Esr1*, possibly as a compensatory mechanism for a loss of ER α activity (Han, Aenlle et al. 2013). Nevertheless, it should be noted that the level of CA1 *Esr1* expression was consistently reduced relative to area CA3, regardless of age or OVX duration.

In other brain regions and tissues, DNA methylation of the ER α promoter is thought to contribute to differential *Esr1* expression. The promoter exon 1b region was chosen for examination as this has been shown to be the active promoter in the rat brain and contains a number of CpG sites previously associated with the regulation of the *Esr1* gene (Freyschuss and Grandien 1996, Champagne, Weaver et al. 2006, Kurian, Olesen et al. 2010). Considerable variability in methylation was observed across the 17 CpG sites located in this promoter region. In general, the greatest methylation was observed at site 1 and minimal methylation was observed for sites 2–10. Modest methylation was observed for sites 11–17 with sites 11, 15, and 17 exhibiting higher methylation relative to upstream sites 2–7.

DNA Methylation is involved in heritable gene silencing or gene inactivation (Bird and Wolffe 1999, Newell-Price, Clark et al. 2000). While promoter regions that are highly methylated tend to be less transcriptionally active, the relationship between DNA methylation and gene expression is far from clear. In the current study, the largest difference in *Esr1* expression was observed across hippocampal subregions and the site of the greatest DNA methylation, site 1, also exhibited differential methylation across subregions, with increased methylation in region CA1 associated with reduced *Esr1* expression. Similarly, the increase in mRNA expression in CA1 with age was associated with decreased methylation of site 15 in CA1 and the increase in *Esr1* for young long-term OVX and aged animals was associated with decreased methylation in CA1 of sites 11 and 14, compared to young short-term OVX animals. The results indicate that for sites with the greatest methylation (i.e. site 1), variability in methylation is associated with changes in *Esr1* expression. Furthermore, for more distal sites (i.e. 11, 14, 15), methylation is more modest and can be modified across the lifespan. Each of these points are addressed below.

The idea that increased methylation of site 1 is related to decreased mRNA expression is consistent with previous work in other brain regions (Kurian, Olesen et al. 2010). However, we also observed increased methylation for sites 14 and 15 in region CA3 associated with increased *Esr1* expression. Similarly, Gore et al. (2011) have reported that exposure to an estrogenic endocrine disruptor increased DNA methylation at one site, identified as site 14 of the *Esr1* promoter in the current study, which was associated with increased mRNA levels in the preoptic area (Gore, Walker et al. 2011). Thus, it should be emphasized that the variability in methylation, increasing or decreasing, only provides a correlate of mRNA

expression. A mechanism through which differential DNA methylation might regulate *Esr1* expression remains to be elucidated.

The idea that methylation of these distal sites can be modified across the lifespan to regulate *Esr1* expression is supported by previous work, which demonstrates that maternal care and hormonal manipulations altered *Esr1* expression in the medial preoptic area and amygdala, and the changes in *Esr1* expression were associated with differential methylation of sites 11–16 (Champagne, Weaver et al. 2006, Edelmann and Auger 2011, Gore, Walker et al. 2011). Across hippocampal subregions there is heterogeneity in DNA methylation, gene expression, and in the transcriptional response to aging (Zeier, Madorsky et al. 2011, Xu 2015). Previous studies have highlighted distinct patterns in DNA methylation and transcription across cell types including different neuronal cell types (Brunner, Johnson et al. 2009, Kozlenkov, Roussos et al. 2014, Angermueller, Clark et al. 2016, Kozlenkov, Wang et al. 2016), suggesting that variability in DNA methylation observed in the current study could be due to cell-type heterogeneity. However, it should also be noted that mRNA for ER α has been observed in both pyramidal cells and interneurons in the hippocampus and expression is higher in region CA3 relative to CA1 (Rune, Wehrenberg et al. 2002). Regardless, it will be important for future studies to determine if the relationship of DNA methylation and *Esr1* expression is specific to certain cell types.

Several transcriptional and post-translational feedback mechanisms control estrogen receptor expression (Bean, Ilanov et al. 2014). However, most of this work has been performed in breast cancer cell cultures and the molecular mechanisms that regulate estrogen receptor expression in the hippocampus are not well understood. In mice, functional knockout of ER α or ER β induces a compensatory increase in hippocampal *Esr1* and *Esr2* transcription, respectively, suggesting a feedback mechanism (Han, Aenlle et al. 2013). The current study suggests that a shift in DNA methylation, particularly for distal sites, could be involved in feedback regulation of ER α expression. Previous studies have reported a link between ER α expression, DNA methyltransferase activity, and ER α promoter methylation during development, aging, and in disease states (Yang, Phillips et al. 2001, Westberry, Trout et al. 2010, Wang, Chou et al. 2012). Methylation of the promoter may influence mRNA expression by regulating the binding of transcription factors. A number of putative transcriptional factors binding sites have been reported for the exon1b region (Gore, Walker et al. 2011), however, only the binding of one transcriptional factor (Stat5b) has been found to be associated with ER α methylation (Champagne, Weaver et al. 2006). In addition, DNA methyltransferase interacts with transcription repressor proteins (e.g. histone deacetylase) to alter chromatin structure and the pattern of DNA methylation (Robertson 2002).

Transcription repressors, Hdac2 and Sap18, influence *Esr1* transcription (Bicaku, Marchion et al. 2008, Ellison-Zelski, Solodin et al. 2009) and E2 treatment decreases the expression of *Hdac2* and *Sap18* in the hippocampus (Aenlle, Kumar et al. 2009). Thus, altered expression of transcription repressor proteins may interact with DNA methylation as part of a feedback mechanism.

Finally, the ratio of hippocampal ER α and ER β expression interacts with the level of E2 to influence transcription and synaptogenesis and a shift in the ER α /ER β ratio may determine the ability of E2 to influence cognition (Hall and McDonnell 1999, Pettersson, Delaunay et

al. 2000, Han, Aenlle et al. 2013, Bean, Ivanov et al. 2014, Bean, Kumar et al. 2015). The increase in *Esr1* and *Esr2* transcription in estrogen receptor knockout mice is diminished for *Esr1*, but not *Esr2*, during aging (Han, Aenlle et al. 2013). It is also interesting to note that E2 treatment of aged animals increases hippocampal synaptic expression of ER β , but not ER α (Waters, Yildirim et al. 2011), suggesting differential regulation of estrogen receptors during aging. Due to the interaction of ER α and ER β in determining functional outcome, it will be important to map out differences in the regulation of these two estrogen receptors during aging.

In summary, transcriptional levels of *Esr1* were altered across hippocampal CA1 and CA3 subregions, with increased expression in region CA3 relative to CA1. In addition, an age-related increase in expression was found in region CA1 relative to young short-term OVX rats. Furthermore, the results support the idea that DNA methylation is an active epigenetic mechanism for the regulation of *Esr1* in the hippocampus, where methylation of site 1 may be the primary regulatory region for cross-regional patterns in ER α expression. Additionally, differential methylation of distal CpG sites, 11–17, was associated with aging or E2 deprivation, suggesting that these sites are modifiable across the life span and may act as a feedback mechanism for ER α activity.

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- Hippocampal ER α promoter methylation associated with *Esr1* expression was examined.
- Methylation of CpG site 1 corresponded to higher mRNA across region CA3-CA1.
- Methylation at sites 11–17 were modifiable across the life span.
- Modifiable sites may act as a feedback mechanism for ER α activity.

Figure 1.

Experimental research design. Young (3 months) and aged (18 months) F344 female rats were handled for 5 minutes a day for 1 week prior to OVX surgery, and sacrificed following a short-term E2 deprivation time (3 weeks) or a long-term E2 deprivation time (14 weeks).

Figure 2.

Illustration of differences in *Esr1* expression. The CA1 region from young short-term rats were used as the calibrator sample in calculating the $\Delta\Delta CT$. For **A–C**, each bar represents the mean (+SEM) of *Esr1* expression for the relevant variable, collapsed across all other variables. *Esr1* expression is increased with **A**) age, **B**) long-term OVX, and **C**) in region CA3 relative to region CA1. Asterisks in **A–C** indicated a significant ($p < 0.001$) main effect. **D**) Examination of interactions, where each bar represents the mean (+SEM) of *Esr1* expression for young ($n=5-6$; open bars) and aged ($n=6$; filled bars), across regions CA1 and CA3 according to OVX duration (short-term: 3 wk, long-term: 14 wk). Asterisk indicates a significant ($p < 0.05$) increase in region CA1 relative to young short-term OVX CA1. Pound sign indicates a significant ($p < 0.05$) increase in region CA3 relative to young short-term OVX CA3. For aged animals, long-term OVX was associated with increased expression relative to aged short-term OVX for region CA1 only (α , $p < 0.05$).

Figure 3.

Site specific DNA methylation of CpG sites across CA1 and CA3 regions. **A)** Each bar represents the mean (+SEM) DNA methylation ratio for sites 1–17 in CA1 (open bars) and CA3 (filled bars). Regional differences in CpG site methylation included increased methylation of site 1 in CA1 and sites 14 and 15 in CA3 (n=20 per region). Asterisk indicates a significant ($p<0.05$) increase in methylation. **B)** Hierarchical clustering and heatmap of the clones which contained 1 methylated site (CA1: 104 clones (51%) & CA3: 84 clones (41%).

Figure 4.

ER α promoter DNA methylation is altered in regions CA1 and CA3 during aging. Age-related differences in CpG site methylation for **A**) area CA1 were observed as an increase in DNA methylation for site 15 for young animals (open bars, n=10) relative to aged animals (filled bars, n=10). **B**) For area CA3, site 17 contained higher methylation in young rats, and sites 11 and 12 showed higher methylation in aged rats. Due to the influence of OVX duration on transcription, young animals were separated into short-term OVX (Young-s) and long-term OVX (young-l) and compared to all aged animals for sites 11–17. Specific differences were observed in CA1 for **C**) site 11 and **D**) site 14. **E**) For region CA3, a difference was observed for site 13. Bars represent the mean (+SEM) of the methylation ratio. Asterisks indicate a significant ($p < 0.05$) increase in **A** and **B** and a significant ($p < 0.05$) difference from young short-term in **C–E**.

Table 1Fisher's PLSD for CpG Sites in *Esr1* promoter

DNA methylation on CpG site	P-Value
1 > 16 sites (2–17)	<0.05
8 > 1 sites (5)	<0.05
10 > 2 sites (5, 9)	<0.05
11 > 8 sites (2–7, 9, 14)	<0.05
12 > 3 sites (3, 5, 9)	<0.05
13 > 3 sites (3–7, 9)	<0.05
15 > 9 sites (2–9, 14)	<0.05
16 > 4 sites (3, 5, 6, 9)	<0.05
17 > 8 sites (2–7, 9, 14)	<0.05