

Sex-Specific Regulation of β -Secretase: A Novel Estrogen Response Element (ERE)-Dependent Mechanism in Alzheimer's Disease

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Women have a higher prevalence and incidence of Alzheimer's disease (AD) than age-matched men, and loss of estrogen might be partially responsible for the higher risk of AD in aged women. While β -secretase (BACE1) plays an important role in AD pathogenesis, whether BACE1 involved the sex difference in AD pathology remains unclear. This study investigated the hypothesis that estrogen regulates BACE1 transcription via the estrogen response element (ERE) and designated pathways. Using estrogen receptor (ER) knock-out mice and mutagenesis of EREs in HEK293 cells, we demonstrated sex-specific inhibition of BACE1 transcription by estrogen via direct binding to ERE sites and ER α . We also used a repressor of estrogen receptor activity (REA) and showed that an REA-ERE complex downregulated BACE1. A chromatin immunoprecipitation assay analysis determined that all three EREs at the BACE1 promoter were required for estradiol-mediated downregulation of BACE1 transcription in mice. Last, we confirmed the impairment of the REA pathway in the cortex of female AD patients. Our study identified an estrogen-specific BACE1 transcriptional regulation pathway from cell and animal models to AD patients.

Key words: Alzheimer's disease; BACE1; estrogen; sex difference

Significance Statement

With the increase in the aging population and Alzheimer's disease worldwide, an urgent need to find effective approaches to treat or prevent AD. Women have a higher prevalence and incidence of AD than men. Identification of the sex-specific risk for AD may be valuable for disease prevention. This study evaluated several estrogen response element (ERE) sites on the promoter of β -secretase (BACE1), a key enzyme for AD pathology. We demonstrated that estrogen downregulated BACE1 transcription through direct binding and complex formation with ERE and cofactors. Our novel findings provide evidence that an estrogen supplement may decrease the risk of AD in menopausal and postmenopausal women. Furthermore, this study demonstrates the "sex-specific" mechanisms of BACE1 as a role in AD pathogenesis.

Introduction

Women have a higher prevalence and incidence of Alzheimer's disease (AD) than age-matched men, even after adjusting for age and education (Baum, 2005; Hebert et al., 2013; Alzheimer's Association, 2017; Snyder et al., 2016). While the reason for the

sex difference in AD prevalence remains uncertain, multiple lines of evidence suggest that the precipitous age-related loss of estrogen in women is partially responsible for the sex-specific risk (Merlo et al., 2017; Vegeto et al., 2020). In preclinical and epidemiological studies, estrogen replacement therapies and genistein are considered protective against AD (Sharifi-Rad et al., 2021). Estrogen replacement is also being evaluated to prevent and treat AD in women (Snyder et al., 2016). We previously demonstrated that deficits in endogenous estrogen, especially deficits in brain-derived estrogen, are associated with the upregulation of β -secretase (BACE1) and increased AD pathology exclusively in female mice only (Yue et al., 2005; McAllister et al., 2010), a finding that others have recently confirmed (Hwang et al., 2016). Others showed that estrogen downregulates BACE1 protein in human

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cell culture (Bernstein et al., 2011; Huang et al., 2020). We have also demonstrated that early estrogen treatments can significantly downregulate BACE1 mRNA and protein levels *in vitro* and *in vivo* (Li et al., 2013). However, whether the estrogen-related BACE1 regulation is one of the sex-specific risk factors of AD in women and the mechanisms underlying estrogen-mediated regulation of BACE1, particularly at the transcriptional level, remains unknown.

BACE1 cleavage of the amyloid precursor protein (APP) is the first step in β -amyloid ($A\beta$) production. Several gene mutations related to the early onset of AD can alter APP processing and $A\beta$ production such as promoting the cleavage at the β -site (N-terminal side of the $A\beta$ sequence) to trigger AD pathologies in affected individuals (Jonsson et al., 2012; Shen et al., 2018). BACE1 is limiting for $A\beta$ production as demonstrated by its reduction in heterozygous knock-out mice (Sadleir et al., 2015). Under normal conditions, the expression level of the BACE1 gene is relatively low because of its weak promoter activity (Li et al., 2006). However, BACE1 gene expression increases with age (Fukumoto et al., 2004; Hampel et al., 2020) and is particularly elevated in the brain cortex in AD patients (Fukumoto et al., 2002; Holsinger et al., 2002; Yang et al., 2003; Ahmed et al., 2010; Das and Yan, 2019). This age- and AD-specific change of BACE1 suggests an important role of BACE1 gene regulation in abnormal APP processing and AD pathology. The promoter of the human BACE1 gene has been cloned and characterized (Christensen et al., 2004; Sambamurti et al., 2004), and several putative transcription factor binding sites were identified, such as sites for GC box, cAMP response element-binding protein (CREB), PU-box, activator protein-1 (AP-1), AP-2, and estrogen response elements (EREs; Christensen et al., 2004; Sambamurti et al., 2004). A variety of transcription factors, including Sp1, Yin Yang 1, and nuclear factor- κ B (NF- κ B), regulate BACE1 gene expression through binding to the specific sites and alter APP processing pathways and $A\beta$ production (Christensen et al., 2004; Nowak et al., 2006; Bourne et al., 2007). Consequently, elevated BACE1 protein and enzymatic activity have been found in the brains of patients with mild cognitive impairment and AD, indicating that BACE1 is important in AD development (Cheng et al., 2014). Interestingly, our recent study showed a similar elevation of BACE1 activity in peripheral nervous system and CSF in mild cognitive impaired (MCI) and AD (Shen et al., 2018), suggesting that BACE1 could be a biomarker in cases of MCI and AD. Therefore, our findings support the notion that targeting and inhibiting BACE1 gene expression could be a valuable anti- $A\beta$ strategy for AD treatment and prevention (Li et al., 2006; Zhou and Song, 2006).

Estrogen exerts regulatory effects on gene expression through multiple nuclear and non-nuclear mechanisms (Gruber et al., 2002; Cui et al., 2013; Krolick et al., 2018). One major mechanism is the direct regulation of transcription by genomic interaction between the estrogen receptor α (ER α) or ER β /ligand complex and specific nuclear DNA target sequences, known as EREs (Hewitt et al., 2010; Cui et al., 2013). In addition to acting directly through EREs, ligand-activated ERs can regulate transcription by a noncanonical pathway through indirect ER/DNA association. Indirect ER-mediated regulation includes interaction with and influence on the activity of other transcription factors, such as SP-1, AP-1, NF- κ B, and c-Jun (Gottsch et al., 2009), which stabilize DNA/protein complexes and recruit coactivators without binding DNA directly.

In this study, we examined the roles of EREs located in the BACE1 promoter (Sambamurti et al., 2004) and identified the specific ER responsible for estrogen-mediated BACE1 transcriptional

Table 1. The primers for subcloning of pGL4.15-mut BACE1P

HiiBACE1.2F	GAATTCGCTAGCCTCACTGCAACCTCTATCTC
ERE1 R	GTGGGTGGATCACCTGGT CTCAGT GGAGTTTGAGACCAGCTGGC
ERE1 F	GCCAGGCTGGTCTCAA ACTCCACTGACC AGGTGTCCACCCAC
ERE1-2jct R	TCGTCTCTCTCAGATTGGTA
ERE1-2jct F	TACCAATCTGAGAGGAGACGA
ERE2 R	GCATATTGTAAGTGCTCAATAA GTCACT GTCTATTAGGATTATTAC
ERE2 F	CAACAGTAATAATCCTAATAG CACTGACC TATTAGGACTTAC
HiiBACE1.2R	GAAAGCAAAGGAATCATTAG
HiiBACE1F	GGAATTCGCTAGCTGTTAGGGAGGTCCTCTTC
ERE3 R	AGCCAAGATGGTCTCGATCT CTCAGAC GTGATCTCCCGCC
ERE3 F	CGAGGGGGAGGATCAC CTGAGG AGATCGAGACCATCTTGG
HiiBACE1R	GGAATTCAGATCTTGGTGGCTCTCAGGAGAG

Table 2. Primers used for amplifying ERE1, ERE2, ERE3, and ERE4 in ChIP assays

ChIP-ERE1 F	ACCAGGCTTTGAGACTCTGT
ChIP-ERE1 R	ACAGGTGTGTACCACTGGAAGT
ChIP-ERE2 F	TCAGATCCACGATGGACACA
ChIP-ERE2 R	TCATTGCCACCCAGAAGACT
ChIP-ERE3 F	CCCTCCACAATGCCAAAGT
ChIP-ERE3 R	GTGGAGTCTACTGGCCTCTT
ChIP-ERE4 F	TGGGCTCCCATTTGATGACA
ChIP-ERE4 R	AACGGATTTGGCCGCTATTGG

repression. Furthermore, we identified a key coregulatory protein that plays a critical role in the ERE/ER complex responsible for estrogen-induced downregulation of BACE1, such as coregulators steroid receptor coregulator 1 (SRC-1) and nuclear coregulator activator 1, which have been reported to interact with estrogen/ERs to regulate gene transcription. We mechanistically investigated the DNA-bound estrogen/coregulator complex associated with estrogen/ERs and, for the first time, found that the estrogen-induced repression of BACE1 transcription is mediated directly by ER α and the ERE/repressor of ER activity (REA) complex. To examine whether BACE1 is a potential target for sex-specific risk of AD, in this study, we investigated the underlying molecular mechanisms of estrogen in BACE1 regulation in *in vivo* and *in vitro* models.

Materials and Methods

Plasmids. The full-length 4 kb human BACE1 promoter region contained three ERE sites in total. It was subcloned from the plasmid pEST-BACE1 P (Sambamurti et al., 2004) as six segments and then ligated into the luciferase reporter vector pGL4.15 (catalog #E670A, Promega; see Fig. 7). The primers listed in Table 1 (from 5' to 3') were used for subcloning. This article labels the most distal ERE as ERE1 and proceeds to the more proximal ones.

Mutations were introduced by direct mutagenesis using the primers ERE1 F/ERE1 R, ERE2 F/ERE2 R, and ERE3 F/ERE3 R. The bold and underlined bases in Table 1 are the nucleotide bases targeted for mutations. Each mutation was subsequently confirmed by sequence analysis, and 0, 1, 2, or 3 of the three mutated sites (ERE1, ERE2, and ERE3) were ligated into the luciferase reporter vector pGL4.15. We subsequently generated several constructs, including a wild-type (WT) BACE1 promoter (pGL4.15-WT BACE1P) and BACE1 promoters with different combinations of mutations (pGL4.15-mut BACE1P).

Animals. Heterozygous ER α and ER β knock-out (ER α ^{+/-} and ER β ^{+/-}, respectively) mice were obtained from The Jackson Laboratory on a C57BL/6NJ background. ER α ^{+/-} mice were crossed with ER α ^{+/-} mice for at least five to seven generations, and the resulting offspring, litter-matched female WT, and ER α ^{-/-} mice were used in the present study. Similarly, heterozygous ER β ^{+/-} mice were crossed with ER β ^{+/-} mice, and litter-matched female WT and ER β ^{-/-} mice were used in the

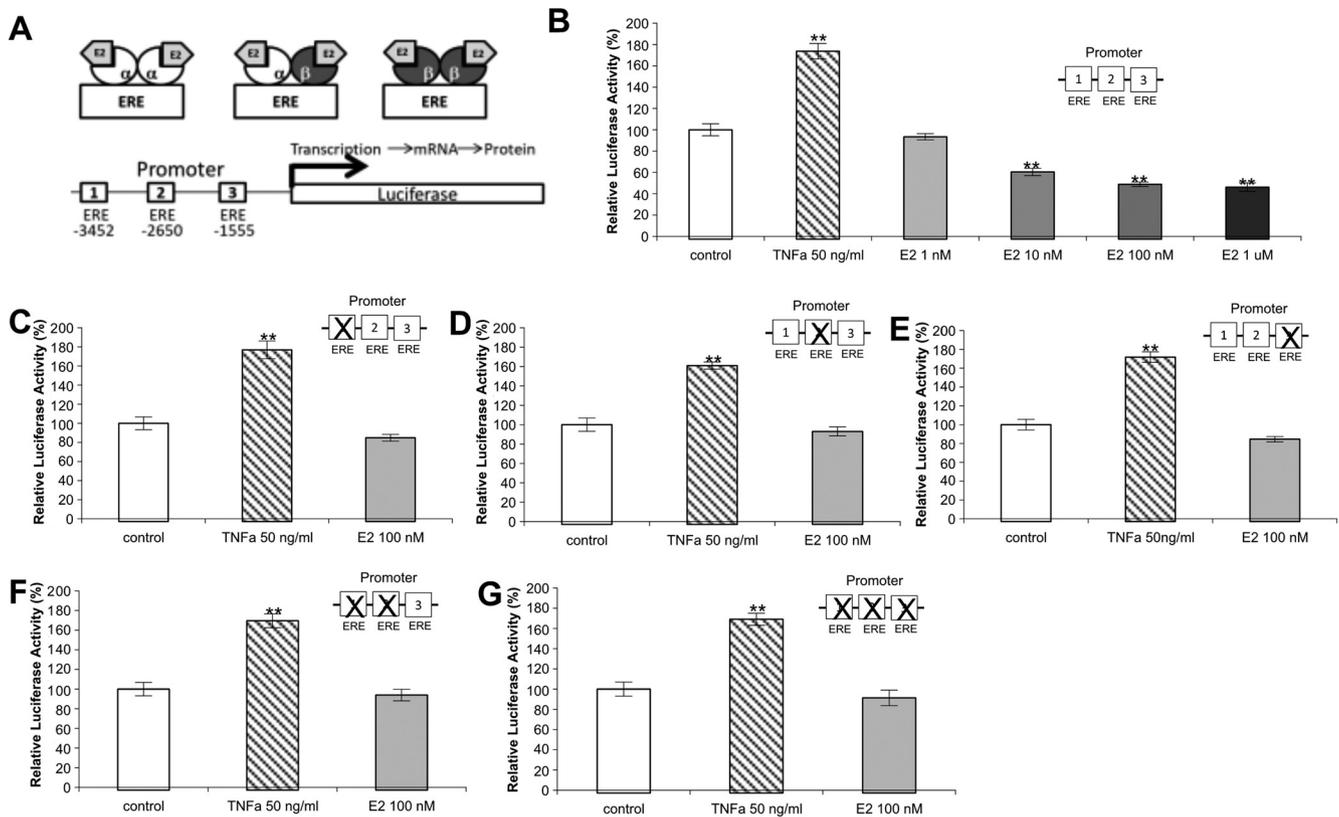


Figure 1. 17 β -Estradiol treatment represses BACE1 promoter activity in an ERE-dependent manner. **A**, BACE1 promoter (wild-type or with different ERE mutations) was introduced into the 5' end of the luciferase gene to drive its transcription in stably transfected HEK293 cells. The positions of the three ERE elements are indicated. EREs act as the binding sites of ligand-activated nuclear estrogen receptors (ER α and ER β) and regulate the transcription of the BACE1 gene. The stable cell lines were treated with either vehicle or different estrogen concentrations, with TNF α as a positive control. Luciferase activity was determined 24 h later. **B–F**, TNF α increased promoter activity in cells transfected with wild-type BACE1 (**B**) and its mutant derivatives with mutant ERE1 (**C**), ERE2 (**D**), ERE3 (**E**), or a combination of mutant ERE1/2 (**F**) or ERE1/2/3 (**G**). E2 treatment repressed the wild-type BACE1 promoter (**B**) but failed to repress Luciferase expression driven by any ERE mutant (**C–G**). In each experiment, data are expressed as a percentage of control (vehicle alone) and are represented as the mean SEM of at least three independent experiments. * $p < 0.05$, versus control; ** $p < 0.01$, versus control.

study. REA knock-out animals were a gift from the laboratory of Benita Katzenellenbogen (University of Illinois, Urbana, IL; Martini and Katzenellenbogen, 2001). Female REA knock-out mice were used in the study. All animal experiments were performed in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the Roskamp Institute and followed the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Estrogen treatments. For *in vivo* studies, 4-month-old female WT, ER $\alpha^{-/-}$, and ER $\beta^{-/-}$ mice were subcutaneously injected with 17 β -estradiol (E2; 20 μ g/50 μ l 10% ethanol/sesame oil) or vehicle (50 μ l 0.10% ethanol/sesame oil) daily for 7 consecutive days. Twenty-four hours after the final injection, mouse brains were collected for Western blot and BACE1 activity assays. For *in vitro* studies, stably transfected HEK293 cells with WT BACE1 promoters or mutated BACE1 promoters were treated with E2 at concentrations of 1 nM, 10 nM, 100 nM, or 1 μ M for 24 h. Then, the cells were treated with 50 ng/ml TNF α (catalog #T6674, Sigma-Aldrich); 1 μ M ICI-182,780 (catalog #Asc-131, Ascent), a high-affinity ER antagonist; 1 μ M 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), a potent, subtype-selective ER agonist (displays selectivity for ER α over ER β); and 1 μ M 2,3-bis(4-hydroxyphenyl) propionitrile (DPN; catalog #Asc-166, Ascent), an ER β -selective agonist.

Cell culture, transfection, and stable cell line development. HEK293 cells were cultured in DMEM (catalog #11995-065, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (#16140-089, Thermo Fisher Scientific) and penicillin/streptomycin (catalog #15140, Thermo Fisher Scientific), and the cells were maintained at 37°C in a 5% CO₂ incubator. pGL4.15-WT BACE1P plasmids and their mutant derivatives (pGL4.15-mut BACE1P) were purified using a commercial kit (catalog #27106, Qiagen) and transfected into HEK293 cells with Lipofectamine 2000 Transfection Reagent (catalog #11668019, Thermo

Fisher Scientific) following the manufacturer instructions. The cells were trypsinized, diluted 1:10, and transferred into fresh growth medium 24 h after transfection. Cells with stably integrated plasmids were selected using a new medium containing 100 μ g/ml hygromycin B (catalog #10 687-010, Thermo Fisher Scientific) after an additional day. The medium was replaced with fresh medium containing hygromycin every 2–3 d to eliminate untransfected cells until the resistant cells formed a polyclonal pool of stably expressing cells. Polyclonal populations stably expressing the WT and mutant forms of pGL4.15-WT BACE1P-luciferase constructs were used for all of the present studies.

Nuclear and cytoplasmic isolation. To prepare nucleus extracts, 4 \times 10⁶ treated cells were trypsinized and incubated for 20 min in a hypo-osmotic buffer containing the following: 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 10 mg/ml aprotinin, 0.5 mg/ml leupeptin, 3 mg/ml PMSF, and 3 mM DTT with 25 ml of 10% NP-40. The nuclei were pelleted by centrifugation for 5 min in a microcentrifuge. The supernatants containing the cytoplasmic proteins were removed and stored at –80°C. We resuspended the pelleted nuclei by gentle shaking in a high-salt buffer containing 50 mM HEPES, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% v/v glycerol, 3 mM DTT, and 3 mM PMSF at 4°C for 30 min to extract the DNA binding proteins. We subsequently removed the nuclei by centrifugation at full speed in a microcentrifuge for 10 min, assayed levels of nuclear proteins in the cleared supernatants, and stored aliquots at –80°C for future assays.

Western blot analysis. Mouse brain tissue was homogenized in M-PER Mammalian Protein Extraction Reagent (catalog #78503, Thermo Fisher Scientific) supplemented with Halt protease and phosphatase inhibitor single-use cocktail (catalog #78442, Thermo Fisher Scientific) and cleared by centrifugation at 1700 \times g for 20 min. The cleared extracts were denatured by boiling in SDS, separated on 8 or 10% SDS-PAGE,

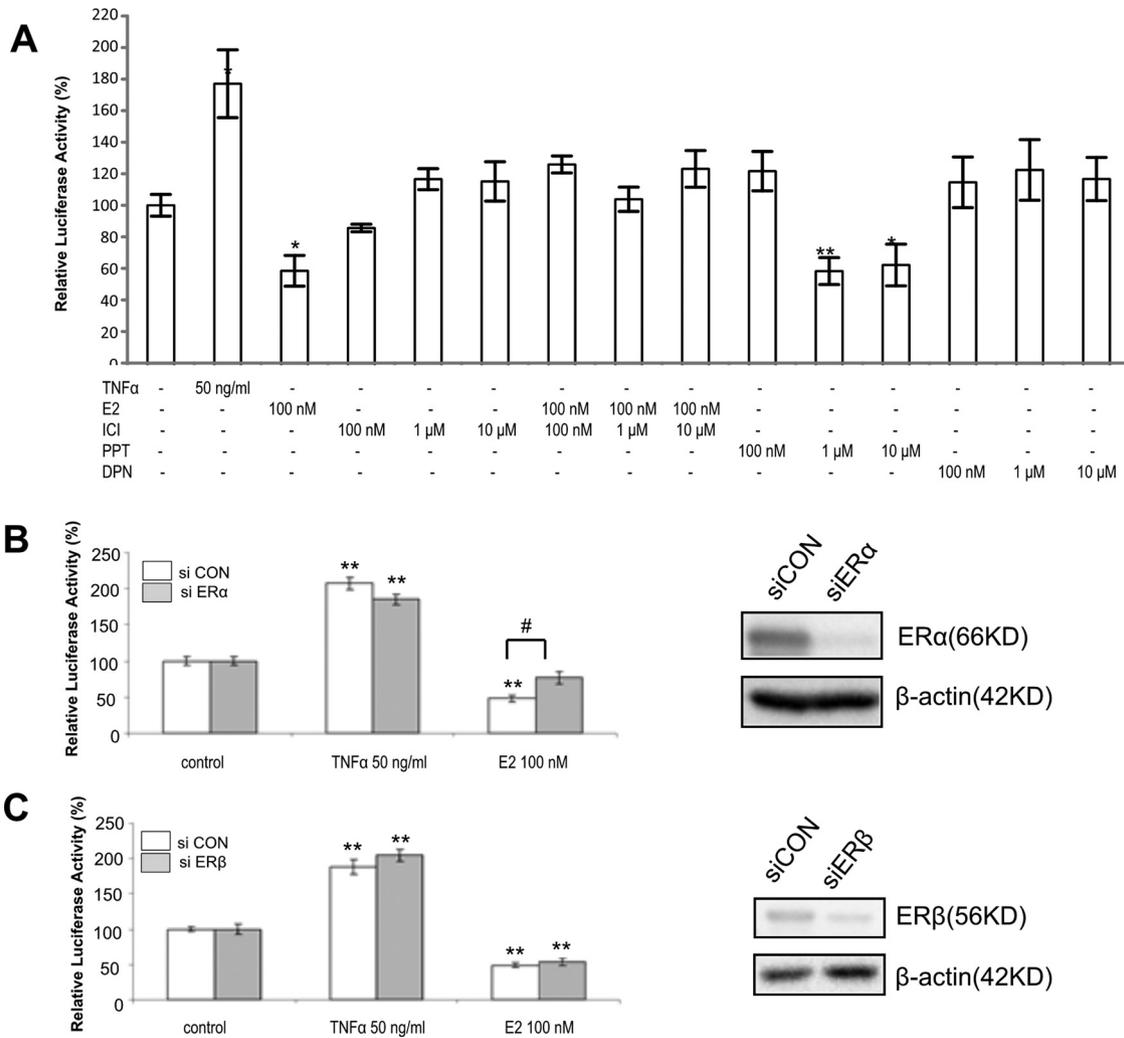


Figure 2. ERα is responsible for E2-induced downregulation of BACE1 promoter activity. **A**, We treated the stable BACE1 wild-type promoter-driven luciferase-expressing HEK293 cell line with a combination of vehicle, TNFα, E2, ICI-182780, PPT, and DPN for 24 h at doses indicated in the label below the x-axis (**A**). Note that TNFα induces BACE1, while E2 or the ERα agonist PPT represses it. The ER antagonist ICI-182780 blocks the E2 repression of BACE1. Unlike the ERα agonist, the ERβ-selective agonist DPN did not affect BACE1 activity. **B**, **C**, We used siRNA to knock down ERα (**B**) or ERβ (**C**) in the same cell line to confirm their relative contribution to BACE1 regulation. Western blots demonstrate the reduction of the two ER receptors after siRNA treatment (**B**, **C**, right panels). Once again, ERα, but not ERβ, knockdown blocked E2-mediated repression of BACE1 promoter activity. Data are expressed as a percentage of control (vehicle alone) normalized to 100% in each experiment and are represented as the mean and SEM of at least three independent experiments. **p* < 0.05, versus control; ***p* < 0.01, versus control; #*p* < 0.05.

transferred to nitrocellulose membranes (catalog #162-0112, BIO-RAD) at 90 mA overnight at 4°C, and blocked with 5% dry milk dissolved in TBS for Western blot analysis.

The membranes were probed with the following primary antibodies overnight: monoclonal mouse anti-BACE1 antibody (1:1000; catalog #MAB931, R&D Systems); rabbit anti-N-terminal BACE1 antibody (catalog #B0806, Sigma-Aldrich); rabbit anti-REA antibody (catalog #A300-658A, Bethyl Laboratories); and rabbit anti-SRC-1 monoclonal antibody (catalog #2191S, Cell Signaling Technology). The membranes were then incubated with corresponding goat anti-mouse or goat anti-rabbit IgG HRP-conjugated secondary antibodies (catalog #SC-2004 and #SC-2055, Santa-Cruz Biotechnology). Pierce ECL Western Blotting Detection Reagent (catalog #32106, Thermo Fisher Scientific) was used for chemiluminescence detection. The signals were detected and captured with a ChemiDoc XRS (BIO-RAD). The membranes were re-probed with mouse anti-β-actin antibody (catalog #A1978, Sigma-Aldrich) after stripping. The signal intensity ratios were quantified by chemiluminescence imaging with Image Lab Software (version 4.6.0; BIO-RAD). The ratios of protein signals to the corresponding β-actin signals were calculated and are expressed as the fold difference between the density ratio of the experimental group ratio and that of the vehicle group.

BACE1 activity assay. An aliquot of brain homogenate was further lysed in lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10% glycerol, and 0.5% Triton X-100). BACE1 enzymatic activity was analyzed as previously reported (Yang et al., 2003; Yue et al., 2005; Zhang et al., 2019) using synthetic peptide substrates containing a BACE1 cleavage site [HK (DABSYL)-SEVNLDAEFRQ(LY)]; catalog #565781, Millipore Life Sciences Research). The BACE1 substrate was dissolved in DMSO and mixed with HAc buffer (100 mM HAc and 100 mM NaCl, pH 4.5). An equal amount of protein was mixed with 100 μl of the substrate. The fluorescence intensity was measured kinetically with a microplate reader (BioTek Instruments) for 2 h at 5 min reading intervals with an excitation wavelength of 430 nm and an emission wavelength of 520 nm. The average reaction velocities were calculated, and the relative velocities of the experimental samples compared with the vehicle samples (100%) were plotted. The BACE1 inhibitor was obtained from Calbiochem (catalog #565788).

Luciferase assay. Twenty-four hours before the luciferase assay, stable cell lines carrying the WT BACE1 promoter or mutated BACE1 promoters were seeded in 96-well culture plates at a density of 40,000 cells/well. After growing the first batch, we froze several aliquots to prevent clonal selection and consequent variations in expression. The luciferase

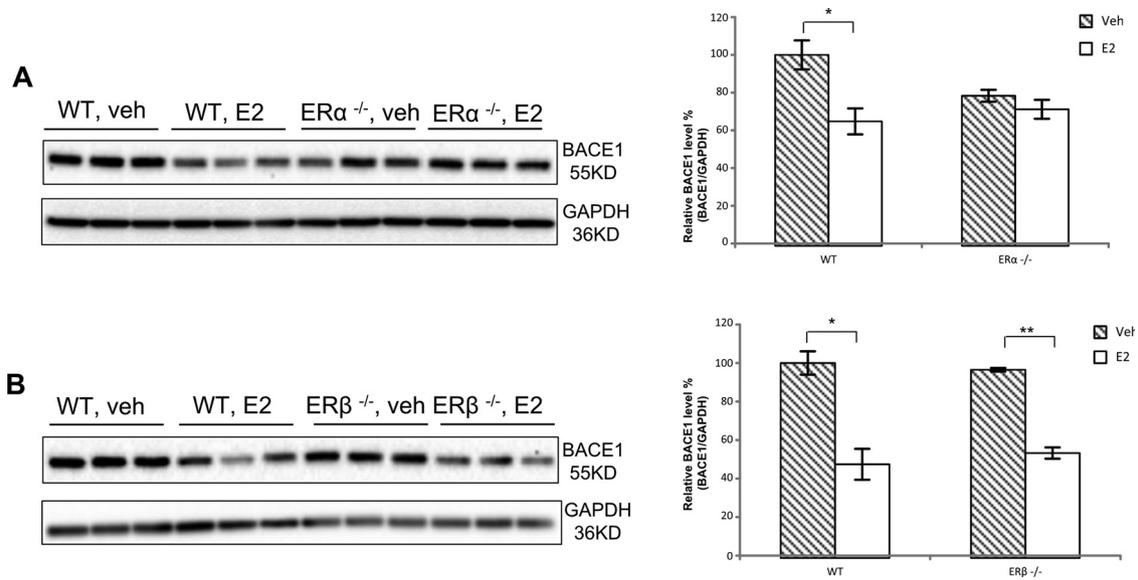


Figure 3. E2 injection can suppress BACE1 protein level in mice brains in an ER α -dependent manner. Wild-type, ER $\alpha^{-/-}$, and ER $\beta^{-/-}$ mice received daily subcutaneous injections of E2 (20 μ g) in 50 μ l of 10% ethanol/sesame oil for 7 d; control animals received vehicle only. **A, B**, Mice were anesthetized, and BACE1 levels in mouse brains were detected. E2 injection suppressed the BACE1 protein level in mouse brain in an ER α -dependent manner. Data are expressed as a percentage of control in each experiment and are represented as the mean \pm SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$. Veh, Vehicle.

assay was performed using a Bright-Glo Luciferase Assay System (catalog #E2620, Promega), following the manufacturer instructions. Luciferase activity was measured using a microplate reader (BioTek Instruments).

Chromatin immunoprecipitation. Brain tissues were minced with a clean razor blade on ice, fixed with 1% formaldehyde for 15 min at room temperature, and quenched for 5 min with glycine added to a final concentration of 0.125 M. The tissues were washed twice with ice-cold PBS and centrifuged at 800 \times g for 5 min. The cell pellets were resuspended in 500 μ l of cell lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Na-deoxycholate, 150 mM NaCl, and protease inhibitor), supplemented with Halt protease and phosphatase inhibitor single-use cocktail (catalog #78442, Thermo Fisher Scientific). The chromatin immunoprecipitation (ChIP) assays were performed essentially following a protocol described previously (Baum, 2005), with minor modifications. Eight micrograms of chromatin was used for each ChIP assay. Before immunoprecipitation, 1% of each sample was removed and saved as input. The samples were incubated with protein A, protein G, and 5 μ g of anti-ER α (catalog #17-603, Millipore) at 4°C overnight; 1 μ g of anti-RNA polymerase II (catalog #GAM-111, SABiosciences) and 1 μ g of mouse IgG (catalog #12-371, Millipore) were used as positive and negative controls, respectively. Finally, we amplified by PCR the immunoprecipitated DNA using the primers listed in Table 2 (from 5' to 3') using the following conditions: 94°C for 10 min; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final step of 72°C for 10 min.

Experimental design and statistical analyses. The data are expressed as the mean \pm SEM. Statistical analysis was performed with ANOVA followed by the least significant difference *post hoc* analysis (for multiple comparisons) or unpaired *t* tests (for pairwise comparisons). Pearson's correlation coefficients were used for correlation analyses. The level of significance was set at $p < 0.05$.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.

Results

Discovery of specific EREs required for E2-induced downregulation of BACE1 transcriptional activity *in vitro*

To investigate whether E2-induced attenuation of BACE1 expression and activity is mediated through EREs, we subcloned the portion of the BACE1 promoter region that contains the

three identified WT EREs in front of the luciferase reporter gene of the vector pGL4.15 (we named this construct pGL4.15-WT BACE1P), as shown in Figure 1A. Then, we introduced mutations into the BACE1 promoter to inactivate the ERE1, ERE2, and ERE3 sequences individually or in different combinations. Stable HEK293 cell lines carrying WT or differently mutated ERE combinations in the BACE1 promoter were established and treated with E2 at concentrations ranging from 1 nM to 1 μ M, as described in Materials and Methods. We examined the E2 responsiveness of the BACE1 promoter by measuring luciferase activity 24 h after E2 treatment, a time point that was chosen based on a time course pilot study (data not shown). TNF α was used as a positive control for stimulation of luciferase expression. As shown in Figure 1B, in WT ERE HEK293 cells, E2 treatment dose-dependently downregulated BACE1 promoter activity with an IC₅₀ of 100 nM compared with vehicle. These data suggest that E2 downregulates BACE1 activity at the transcriptional level. To further examine whether the specific EREs in the BACE1 promoter are responsible for E2-induced transcriptional regulation of BACE1, we treated stable HEK293 cell lines carrying different ERE mutations on the BACE1 promoter with E2 at 100 nM, as shown in Figure 1C–G. Mutations in any one of the three ERE elements led to a loss of repression by E2, indicating that all three identified ERE sites are essential for E2 regulation of BACE1 transcription. Therefore, the three ERE sites appear to cooperate to achieve E2 binding and BACE1 transcriptional repression.

ER α , not ER β is responsible for the E2-induced downregulation of BACE1 promoter activity *in vitro*

To identify which ERs were specifically involved in the inhibitory effect of E2 on BACE1 promoter activity, we performed two *in vitro* studies with pharmacological and molecular interference approaches, respectively. First, we treated HEK293 stable cell lines carrying the WT BACE1 promoter with ICI-182780 (1 μ M), a complete antagonist for both ER α and ER β , for 5 h before treating the cells with E2 (100 nM) or vehicle. As shown in Figure 2A, while cells treated with E2 alone showed significant

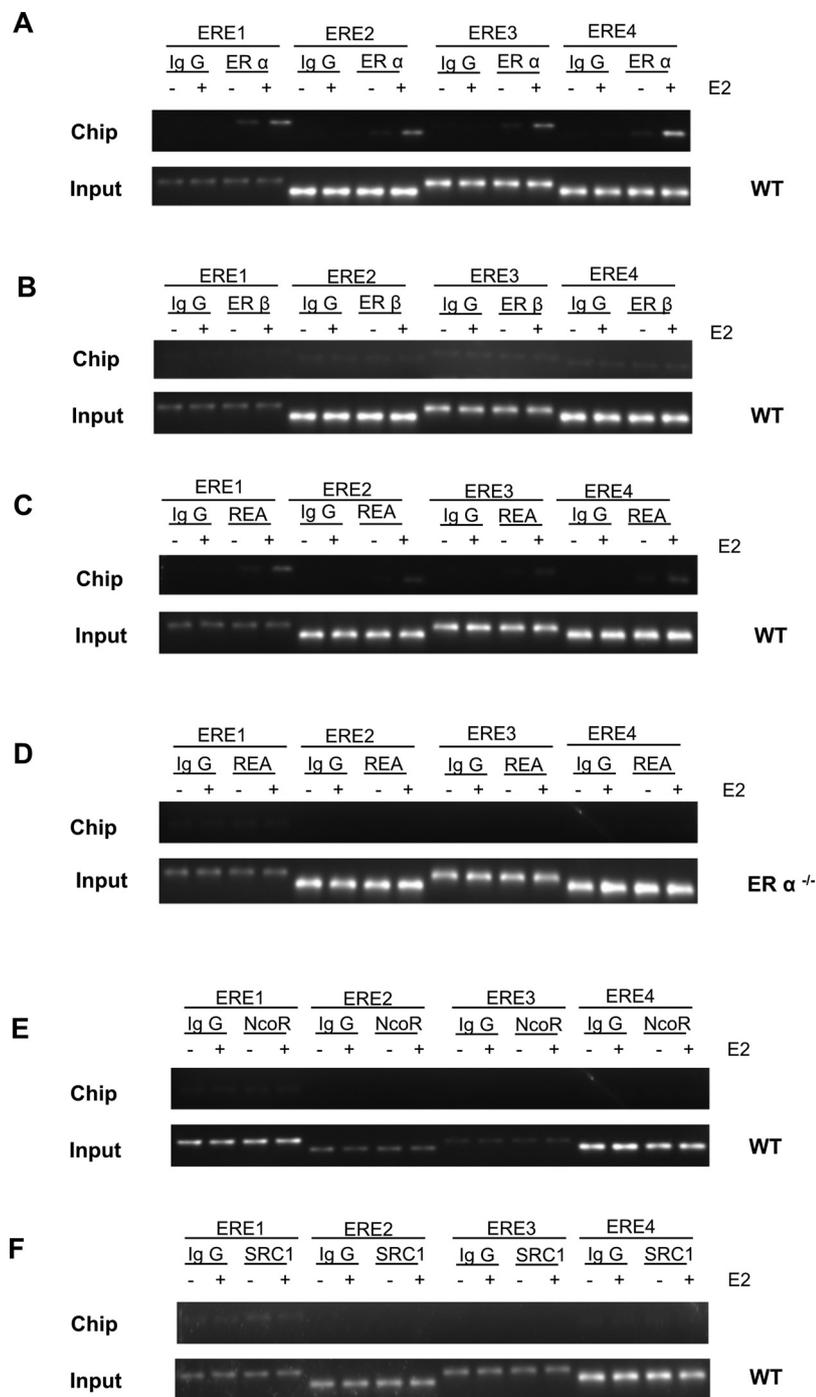


Figure 4. The effect of estrogen on interactions between EREs in BACE1 promoter and ER α , ER β , REA, NcoR, and SRC-1 were detected by ChIP. Wild-type or ER $\alpha^{-/-}$ mice received daily subcutaneous injections of E2 (20 μ g) in 50 μ l of 10% ethanol/sesame oil for 7 d; control animals received vehicle only. Mice were anesthetized, and ChIPs were performed using mouse brain samples. **A**, ER α interacts with ERE1, 2, 3, and 4 in WT mouse brains. **B**, ER β does not interact with ERE1, 2, 3, and 4 in WT mouse brains. **C**, REA interacts with ERE1, 2, 3, and 4 in WT mouse brains. **D**, REA does not interact with ERE1, 2, 3, and 4 in ER $\alpha^{-/-}$ mouse brains. **E**, NcoR does not interact with ERE1, 2, 3, and 4 in WT mouse brains. **F**, SRC-1 does not interact with ERE1, 2, 3, and 4 in WT mouse brains.

downregulation of BACE1 promoter activity, this BACE1 promoter repression was significantly blocked by ICI-182780 pretreatment. These data indicate that E2 suppression of BACE1 transcriptional activity requires the involvement of the classic ER pathway. To further investigate which specific ER (ER α , ER β , or both) was involved in the transcriptional regulation of BACE1, cells were treated with 1 μ M PPT, an ER α -selective ER agonist,

or 1 μ M DPN, an ER β -selective ER agonist. As shown in Figure 2A, compared with vehicle, PPT could significantly suppress BACE1 promoter activity, mimicking the effect of E2; in contrast, DPN did not affect BACE1 promoter activity. The results indicate that ER α is responsible for E2-mediated suppression of BACE1 transcriptional activity.

To further verify the involvement of ER α in E2-mediated suppression of BACE1 transcriptional activity, we transiently knocked down ER α or ER β with small interfering RNAs (siRNAs) in a stable 293 cell line carrying the BACE1 WT promoter. The efficiency of ER knock-down was confirmed by Western blot analysis, which showed reductions in ER α and ER β levels in cells transfected with si-ER α and si-ER β , respectively, compared with cells transfected with si-control (Fig. 2B,C, right). Similar to our data from BACE1 WT promoter-expressing cells pretreated with ICI-182780, we found no reduction in BACE1 promoter activity in si-ER α -transfected (ER α knock-down) cells after E2 treatment (Fig. 2B, left). In contrast, the effect of si-ER β was comparable to that of si-control with regard to E2-induced downregulation of BACE1 promoter activity (Fig. 2C, left). These data further confirm our initial findings and provide direct evidence that the transcriptional regulation of BACE1 by E2 is dependent on the involvement of ER α .

Evidence that genetic depletion of ER α abolishes the E2-induced reduction in BACE1 protein levels in ER $\alpha^{-/-}$ mice further support the ER α specificity of the mechanism of estrogen *in vivo*

To further investigate the specific ER α dependency of the E2-induced inhibition of BACE1 *in vivo*, we conducted an *in vivo* study and examined the levels of BACE1 protein in brains from wild-type, but not ER $\alpha^{-/-}$ mice. However, E2 treatment reduced BACE1 protein expression levels in WT and ER $\beta^{-/-}$ mouse brains (Fig. 3B). We did not observe the E2-mediated repression of BACE1 expression in the liver, suggesting that this regulation is brain specific (see Fig. 8). These data support the ER α dependency of the E2-induced inhibitory effects on brain BACE1 protein.

Identification of E2-induced recruitment of endogenous ER α to the EREs on the mice BACE1 promoter *in vivo*

To understand the ER α -dependent mechanism by which E2 downregulates BACE1 transcriptional activity, we examined the interaction of ER α with EREs in the BACE1 promoter in mice.

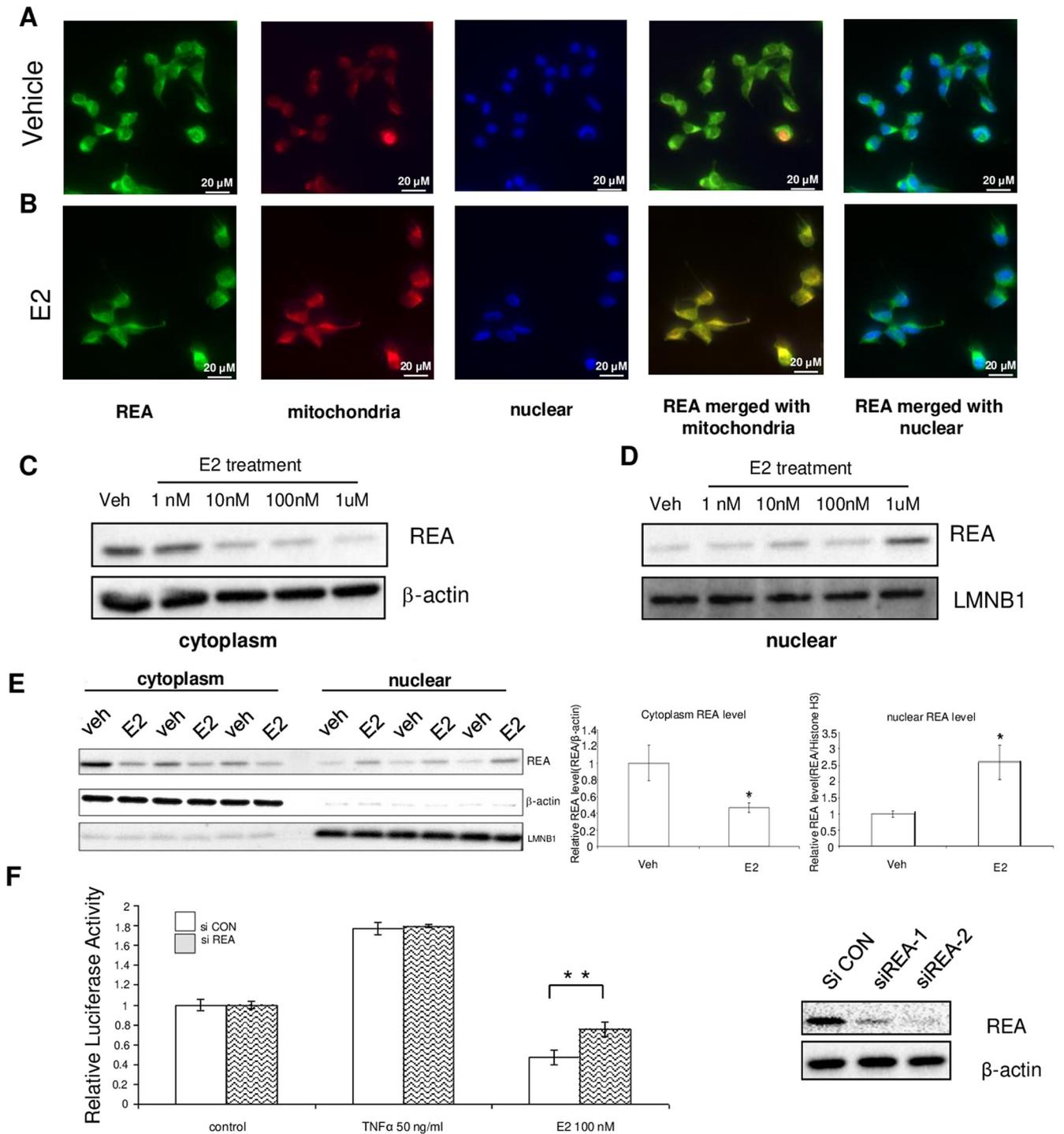


Figure 5. REA translocates from cytoplasm to nuclear and functions as a repressor of BACE1 promoter activity in the presence of E2 in SH-SY5Y cells. **A**, REA is primarily located in the cytoplasm of SH-SY5Y cells, where they colocalize with Mito-tracker Red when treated with vehicle. **B**, Part of REA translocates to the nuclear when treated with 100 nM E2 in SH-SY5Y cells. **C**, **D**, E2 treatment induces the translocation of REA from cytoplasm to nuclear in an E2 dosage-dependent manner in SH-SY5Y cells. **E**, E2 treatment induced translocation of REA from the cytoplasm to the nuclear in the mouse brain. **F**, REA was knocked down using two different siRNAs (siREA-1 and siREA-2) in stable HEK293 cell lines with BACE1 wild-type promoters. The cells transiently transfected with siREA-2 and si-control (Si-CON) were treated with estrogen, with TNF α as a positive control. REA knockdown attenuated the E2-mediated repression of BACE1 promoter activity. Data are expressed as a percentage of control in each experiment and are represented as the mean SEM of at least three independent experiments. ** $p < 0.01$. Veh, Vehicle.

First, in the mice BACE1 promoter region, we searched for ERE sites using TESS software and identified four half-ERE sites located within 4 kb upstream of the transcription start site of the mouse BACE1 promoter; specifically, these sites were located at -3465 , -2752 , -2450 , and -2141 nt upstream of the ATG start codon of the mouse BACE1 gene. Second, to examine the direct binding of each ERE site with ERs, we performed CHIP with

antibodies against ER α or ER β using chromatin isolated from the brains of mice treated with E2 or vehicle. Our data demonstrated that E2 administration significantly promoted the binding of ER α with EREs compared with vehicle treatment, as shown in Figure 4A, indicating that E2 treatment induces the recruitment of ER α to the EREs in the BACE1 promoter *in vivo*. In contrast, as shown in Figure 4B, ER β did not interact with any ERE in the BACE1

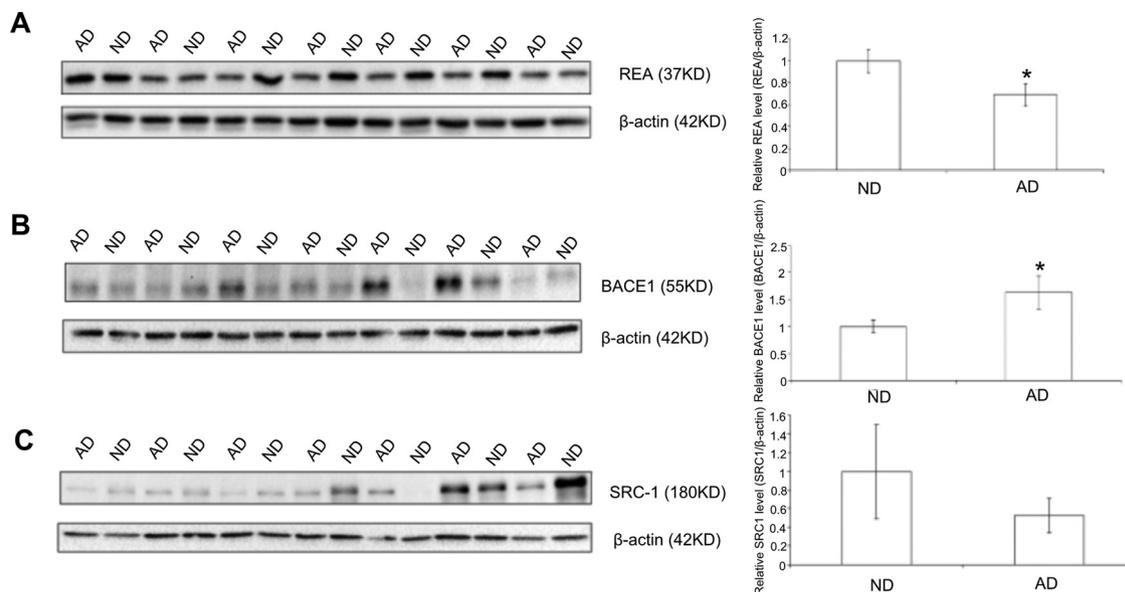


Figure 6. Human AD brains display reduced REA and SRC-1, and increased BACE1 levels. **A–C**, The human brain tissues (cortex) of AD ($n = 7$) and ND ($n = 7$) were homogenized, and Western blot was performed to detect the levels of REA (**A**), BACE1 (**B**), and SRC-1 (**C**). ND, Non demented.

promoter, with or without E2 injection. These data indicate that ER α mediates the regulatory effects of E2 on mouse BACE1 transcriptional activity.

Identification of REA as a transcriptional corepressor required for E2-mediated downregulation of BACE1 transcriptional activity

We then further investigated how the association of ER α with EREs on the BACE1 promoter triggers negative regulation of BACE1 promoter activity. Specifically, we sought to examine whether there was a corepressor. If so, we would identify which corepressor participates in ER-mediated repression of BACE1 transcription. ChIP assays were performed to investigate the potential interactions of corepressors with the BACE1 gene promoter at ERE sites associated with E2-bound ER α . Of all the corepressors tested, only REA was associated with ERE sites within the BACE1 promoter. As shown in Figure 4C, in vehicle-treated mouse brains, we found that REA was weakly associated with four EREs of the BACE1 promoter. However, E2 injection significantly enhanced the interaction of REA with EREs in WT mouse brains. Interestingly, as shown in Figure 4D, in ER α knock-out mice, REA failed to associate with EREs in the BACE1 promoter, demonstrating that the recruitment of REA to EREs in the BACE1 promoter is dependent on ER α . Figure 4, E and F, shows that other classic nuclear receptor coregulators, such as the corepressor NCoR and the coactivator SRC-1, do not interact with EREs in the BACE1 promoter in the mouse brain, with or without E2 treatment. In combination, these data indicate that the recruiting of corepressor REA to EREs in the BACE1 promoter is in an ER α -dependent manner, suggesting the potential effect that REA might exert in the E2-mediated repression of BACE1 gene expression.

It has been reported that REA is predominantly expressed in the mitochondria of HeLa cells, which translocate to the nucleus in the presence of ER α and E2 to function as a repressor of transcription (Kasashima et al., 2006). In this study, we performed immunostaining to confirm REA localization and translocation induced by E2 in SH-SY5Y cells. As shown in Figure 5A, in the absence of E2 treatment, REA was mainly present in the

cytoplasm, colocalized with MitoTracker Red in SH-SY5Y cells. Figure 5B shows that E2 treatment induced the partial nuclear translocation of REA. To further verify whether E2-induced REA translocation into the nucleus, a subcellular fractionation experiment was performed. The cytoplasmic and nuclear fractions of SH-SY5Y cells were purified and monitored with relevant marker proteins. As shown in Figure 5, C and D, E2 induced the translocation of REA from the cytoplasm to the nuclear in neuro-like cells in a dose-dependent manner. A similar experiment was also performed with WT mouse brains with or without E2 injection. As shown in Figure 5E, E2 treatment induced marked translocation of REA from the cytoplasm to the nuclear in the mouse brain. To confirm the function of REA in E2-induced downregulation of BACE1 transcription, we transfected SY5Y cells with two specific siRNAs for REA and subsequently observed depletion of REA (Fig. 5F, right). The cells transfected with si-control or si-REA2 were then treated with E2 at 100 nM. Luciferase activity was determined 24 h after the treatments. As shown in Figure 5F (left), REA depletion abolished the E2-induced downregulation of BACE1 promoter activity, indicating that REA plays an essential role as a corepressor of ER α .

To determine whether low REA levels in the brain are a risk factor for AD, we measured REA levels in postmortem brain samples from 7 female AD patients and 7 female non-AD control subjects. We also detected BACE1 protein levels in the same AD patients and non-AD subjects. As shown in Figure 6, A and B, the brains of AD patients had significantly reduced REA levels. However, they significantly increased BACE1 levels compared with non-AD subjects, suggesting that REA levels are deficient in AD brains. We also examined the levels of the nuclear receptor coactivator SRC-1 in the same AD patients and non-AD subjects. The results showed no significant difference in the SRC-1 levels between the two groups (Fig. 6C).

Discussion

In the present study, we investigated the molecular mechanisms by which estrogen regulates BACE1 activity *in vivo* and *in vitro*.

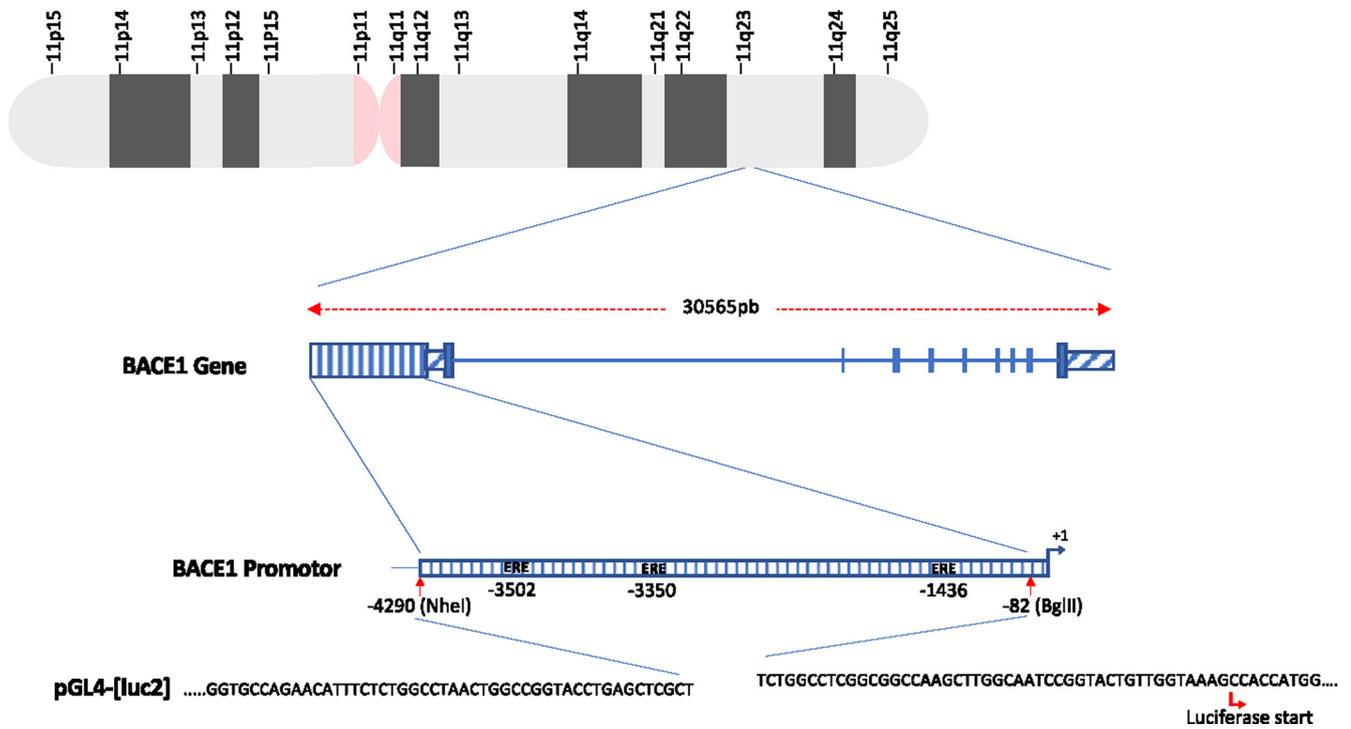


Figure 7. Cloning strategy of pGL4.15-BACE1P. The 4 kbp BACE1 promoter was cloned into the pGL4 vector between the restriction sites NheI and BglII.

For the first time, our results demonstrated that estrogen-induced downregulation of BACE1 activity requires functional ER α as well as three upstream half-palindromic ERE (5'-TGACCT-3') motifs in the BACE1 promoter.

Moreover, we have confirmed that E2 can significantly downregulate BACE1 at the transcriptional level and found that the E2-mediated regulation of BACE1 promoter activity depends on each specific ERE site located on the BACE1 promoter. Because EREs may exist as single or multiple sites and may be flanked by other transcription factors (e.g., Sp1), we performed mutagenesis studies to identify the role of each TGACCT-box half-ERE in the BACE1 promoter. We found that mutation of any one of the EREs in the BACE1 promoter completely abolished estrogen-induced regulation of BACE1 transcriptional activity. These results suggest that three widely separated ERE half-palindromic motifs mediate the transcription of BACE1.

Highly estrogen-responsive and perfectly palindromic sequences have been found in the African clawed frog (*Xenopus laevis*) genes encoding vitellogenin A1, A2, B1, and B2; these sequences are composed of two hexameric core half-site motifs of 5'-TGACCT-3' with a spacer of 3 bp (Klein-Hitpass et al., 1988; Anolik et al., 1995). However, in the human genome, most estrogen target genes do not contain an ERE palindrome in their promoters but rather have nonpalindromic EREs (half-EREs) through which estrogen regulation is mediated (Anolik et al., 1995). Although half-EREs confer only weak estrogen

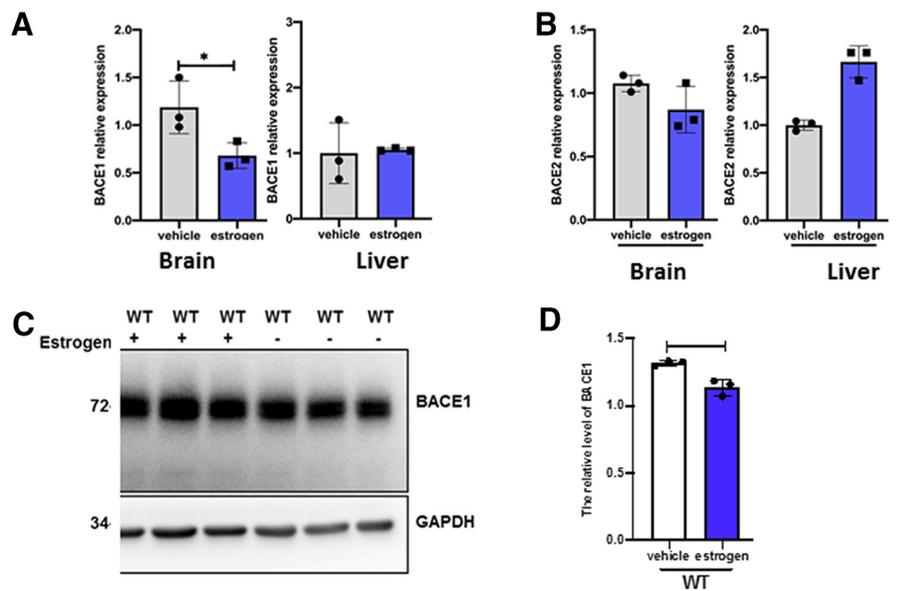


Figure 8. Estrogen treatment reduces the level of BACE1 in the brain, but not in the liver of C57BL/6J mice. **A, B**, BACE1 RNA (**A**) in the brain and liver (**B**, but also in **A**) measured after treatment with vehicle alone (50 μ l of 10% ethanol/sesame oil) or 20 μ g of E2. BACE2 RNA from the same tissue (**B**). **C, D**, Western blot shows brain BACE1 protein (**C**) with quantification (**D**) after E2 treatment. Note that BACE1 protein (apparent molecular weight, 72 kDa in MOPS gels) is not detected in the mouse liver (www.biogps.org).

responsiveness, several genes have been described for which transcription is induced by two or more ERE half-sites (Anolik et al., 1995). For instance, the promoter of the human CRH gene contains four half-ERE sites that have been shown to respond to estrogen administration (Vamvakopoulos and Chrousos, 1993). In addition, prothymosin α gene expression is increased by estrogen via two half-palindromic EREs located in the 5 kb promoter region (Martini and Katzenellenbogen, 2001). In the present study, we have shown, for the first time,

that the three half-ERs in the BACE1 promoter act synergistically to mediate E2-induced regulation of BACE1 transcription.

While both ER α and ER β are widely distributed and expressed in neuronal and non-neuronal cell types, the transcriptional effects of estrogen are mainly ER α and ER β dependent (Couse and Korach, 1999). Despite their similarities, ER α and ER β are structurally and functionally distinct in many respects, such as in ligand recognition, receptor activation, and coregulator recruitment, as well as in the target genes they regulate (Couse and Korach, 1999). An extensive body of literature has documented genes regulated specifically by E2/ER α interactions (Kim et al., 2011). However, it is unclear whether ERE-induced repression of BACE1 has ER specificity. In the current study, using selective ER agonists and siRNAs, we have shown that ER α , but not ER β , is responsible for the estrogen-induced downregulation of BACE1 promoter activity. This study is the first to demonstrate that estrogen-induced BACE1 downregulation is mediated through ER α . Using ChIP techniques, we also demonstrated that E2 induced direct recruitment of ER α , but not ER β , to the EREs in the BACE1 promoter in the mouse brain, further supporting the ER α dependence of the mechanism by which E2 regulates BACE1.

Ligand-bound ERs can regulate the expression of target genes either directly by binding to specific EREs in the promoter regions of target genes or indirectly by tethering to certain other transcription factors, such as AP-1 (Paech et al., 1997; Webb et al., 1999), Sp1 (Safe, 2001; Safe and Kim, 2004), p53 (Liu et al., 2006; Sayeed et al., 2007) and NF- κ B (De Bosscher et al., 2006). To understand the mechanism by which the association of ER α with EREs on the BACE1 promoter triggers negative regulation of BACE1 promoter activity, we examined the binding of several corepressors with EREs in the BACE1 promoter. It is well established that ER shares properties with other steroid hormone receptors; for example, the positive or negative regulatory effects of ERs on target genes are determined by the specific coactivators or corepressors recruited to particular promoters in the target tissues. Coactivators or corepressors can activate or repress transcription, respectively, by further recruiting other elements associated with chromatin remodeling or transcriptional machinery (Aranda and Pascual, 2001; Nilsson et al., 2001; Dobrzycka et al., 2003). To date, most of the identified coregulators of ERs are coactivators, including, most prominently, SRC-1 (Hussain et al., 1999) and related p160 family members (Sinha et al., 1999) as well as CREB (Vassar et al., 1999).

Interestingly, CREB is involved in cell type-specific signaling pathways in response to the activation of ERs. It has been reported that the activation of ERs triggers different rapid signaling pathways in neurons and astrocytes. In neuronal cells, the activation of ERs is coupled to the activation of CREB. Conversely, in astrocytes, E2 instead activates signaling pathways leading to the inhibition of CREs and Cre-mediated transcription (Roque and Baltazar, 2019). Previous studies have also focused on the mechanisms that lead to the inhibition of target gene expression, which has implicated the classic nuclear receptor corepressors NCoR and SMRT as well as the ER-specific corepressor REA in transcriptional repression (Shang and Brown, 2002; Liu and Bagchi, 2004; Métiévier et al., 2004). Our results show that REA is responsible for E2-induced repression of BACE1 gene expression, providing a potential therapeutic target for AD. Additionally, the REA deficiency in the nuclear fraction of human AD brains may explain the previously reported upregulated BACE1 protein levels and enzyme activity (Yue et al., 2005).

Our studies still have some caveats. For example, although our current experimental data showed that E2-induced downregulation of BACE1 promoter activity could be blocked by ER antagonist ICI-182780, PPT (ER α antagonist), and si-ER α , but not DPN (ER β antagonist) or si-ER β (Fig. 2, revised), an additional ER agonist or activator might be another layer of validation for the specificity of ER β .

A second limitation is that we did not include male and aged mice to study the interaction between estrogen receptors and BACE-ERE in this investigation. Our initial studies in male subjects (Yue et al., 2005; McAllister et al., 2010) suggest that the AD-associated BACE1 increase is seen only in female subjects (Figs. 6, 7, 8). Another limitation is that we did not include male and aged mice to study the interaction between estrogen receptors and BACE-ERE in this investigation. Furthermore, we expect to evaluate the sex-specific correlation between REA and AD biomarkers in future follow-up studies. Nevertheless, the studies support E2 control of BACE1 by ER α , not ER β , using multiple methods in female mice.

In this study, the mechanisms involved in E2-mediated downregulation of BACE1 gene expression were examined. For the first time, we report that estrogen formed a complex with ER α and REA by directly binding to three upstream half-palindromic ERE motifs (5'-TGACCT-3') within the BACE1 promoter to downregulate BACE1 transcription. The ability of E2-bound ER α and REA to suppress BACE1 expression indicates a positive role for the corepressor REA in the regulation of A β production in AD. We propose that BACE1 induction following the drop in estrogen levels may increase the risk of AD in women. Estrogen replacement with A β measurements may offer a paradigm to mitigate AD risk while limiting estrogen-mediated adverse events.

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